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Towards defining the chloroviruses: a genomic journey through a genus of large DNA viruses

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Abstract

Background: Giant viruses in the genus *Chlorovirus* (family *Phycodnaviridae*) infect eukaryotic green microalgae. The prototype member of the genus, *Paramecium bursaria* chlorella virus 1, was sequenced more than 15 years ago, and to date there are only 6 fully sequenced chloroviruses in public databases. Presented here are the draft genome sequences of 35 additional chloroviruses (287 – 348 Kb/319 – 381 predicted protein encoding genes) collected across the globe; they infect one of three different green algal species. These new data allowed us to analyze the genomic landscape of 41 chloroviruses, which revealed some remarkable features about these viruses.

Results: Genome colinearity, nucleotide conservation and phylogenetic affinity were limited to chloroviruses infecting the same host, confirming the validity of the three previously known subgenera. Clues for the existence of a fourth new subgenus indicate that the boundaries of chlorovirus diversity are not completely determined. Comparison of the chlorovirus phylogeny with that of the algal hosts indicates that chloroviruses have changed hosts in their evolutionary history. Reconstruction of the ancestral genome suggests that the last common chlorovirus ancestor had a slightly more diverse protein repertoire than modern chloroviruses. However, more than half of the defined chlorovirus gene families have a potential recent origin (after Chlorovirus divergence), among which a portion shows compositional evidence for horizontal gene transfer. Only a few of the putative acquired proteins had close homologs in databases raising the question of the true donor organism(s). Phylogenomic analysis identified only seven proteins whose genes were potentially exchanged between the algal host and the chloroviruses.

Conclusion: The present evaluation of the genomic evolution pattern suggests that chloroviruses differ from that described in the related *Poxviridae* and *Mimiviridae*. Our study shows that the fixation of algal host genes has been anecdotal in the evolutionary history of chloroviruses. We finally discuss the incongruence between compositional evidence of horizontal gene transfer and lack of close relative sequences in the databases, which suggests that the recently acquired genes originate from a still largely un-sequenced reservoir of genomes, possibly other unknown viruses that infect the same hosts.

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Background

Viruses in the family *Phycodnaviridae*, together with those in the *Poxviridae*, *Iridoviridae*, *Ascoviridae*, *Asfarviridae* and the *Mimiviridae* are believed to have a common evolutionary ancestor and are referred to as nucleocytoplasmic large DNA viruses (NCLDV) [1-3]. Members of the *Phycodnaviridae* consist of a genetically diverse, but morphologically similar, group of large dsDNA-containing viruses (160 to 560 kb) that infect eukaryotic algae [4,5]. These large viruses are found in aquatic environments, from both terrestrial and marine waters throughout the world. They are thought to play dynamic, albeit largely undocumented roles in regulating algal communities, such as the termination of massive algal blooms [6-8], which has implications in global geochemical cycling and weather patterns [9].

Currently, the phycodnaviruses are grouped into 6 genera, initially based on host range and subsequently supported by sequence comparison of their DNA polymerases [10]. Members of the genus *Chlorovirus* infect chlorella-like green algae from terrestrial waters, whereas members of the other five genera (*Coccolithovirus*, *Phaeovirus*, *Prasinovirus*, *Prymnesiovirus* and *Raphidovirus*) infect marine green and brown algae. Currently, 24 genomes of members in four phycodnavirus genera are present in Genbank. Comparative analysis of some of these genomes has revealed more than 1000 unique genes with only 14 genes in common among the four genera [4]. Thus gene diversity in the phycodnaviruses is enormous.

Here we focus on phycodnaviruses belonging to the genus *Chlorovirus*, referred to as chloroviruses (CV). These viruses infect certain unicellular, eukaryotic, exsymbiotic chlorella-like green algae, which are often called zoochlorellae; they are associated with either the protozoan *Paramecium bursaria*, the coelenterate *Hydra viridis* or the heliozoon *Acanthocystis turfacea* [11]. Three such zoochlorellae are *Chlorella* NC64A, recently renamed *Chlorella variabilis* [12], *Chlorella* SAG 3.83 (renamed *Chlorella heliozoae*) and *Chlorella* Pbi (renamed *Micratinium conductrix*). Viruses infecting these three zoochlorellae will be referred to as NC64A-, SAG-, or Pbi-viruses.

Since the initial sequencing of the prototype CV, *Paramecium bursaria* chlorella virus 1 [13,14], more than 15 years ago, only 5 more whole-genome sequences of CVs have been reported [15-17]. These 6 sequences reveal many features that distinguish them from other NCLDV including genes encoding a translation elongation factor EF-3, enzymes required to glycosylate proteins [18], enzymes required to synthesize the polysaccharides hyaluronan and chitin, polyamine biosynthetic enzymes, proteins that are ion transporters and ones that form ion channels including a virus-encoded K^+ channel (designated Kcv) [19], a SET domain-containing protein (referred to as vSET) that dimethylates Lys^{27} in histone 3 [20,21], and many DNA methyltransferases and DNA site-specific endonucleases [22,23]. Moreover, the evolution of large DNA viruses is subject to intense debate. Questions include, how did this vast gene diversity arise? Are genes captured from organisms or viruses, or did genome reduction occur from a larger ancestor? Here we address these questions by sequencing and comparing the genomes of 41 CVs infecting 3 different green algal species.

Results and discussion

Terrestrial water samples have been collected throughout the world over the past 25 years and plaque-assayed for CVs. The viruses selected for sequencing (Figure 1) were chosen from a collection of more than 400 isolates with the intention of evaluating various phenotypic characteristics and geographic origins as indicators of diversity; an equal number of isolates infecting each of the three hosts were selected. However, this selection of viruses does not represent a biogeographic survey.

The viral genomes were assembled into 1 to 39 large contigs (with an average length of 40 Kb), had cumulated sizes ranging from 287 to 348 Kb and an average read coverage between 27 and 107 (Table 1). Contig extremities often contained repeated sequences that interfered with the assembly process and precluding obtaining a single chromosome contig. Two virus assemblies contained a large number of contigs – i.e., Fr5L and MA-1E containing 22 and 39 contigs respectively. In fact, >90% of the Fr5L and MA-1E sequences were contained in 5 and 9 large contigs, respectively, which is similar to the number of large contigs in the other virus assemblies. The remaining contigs were small (<1 kb for the majority) and showed strong sequence similarity with reference genomes, which suggests that they did not arise from contamination. Like the previously sequenced CVs, the G + C content of the newly sequenced genomes was between 40% and 52%. Moreover, the G + C content was highly homogeneous and specific among viruses infecting the same host: i.e., NC64A, Pbi and SAG viruses had a median G + C content of 40%, 45% and 49%, respectively with low standard deviation in each group (<0.14%).

Gene prediction algorithms identified 319 to 381 protein-encoding genes (CDSs) in each genome, of which 48% were given a functional annotation. Furthermore, each genome was predicted to contain between 5 and 16 tRNA genes. These features resemble the 6 previously sequenced CV genomes that had 329 to 416 protein-encoding genes and 7 to 11 tRNA genes [14-16]. However, we cannot rule out the possibility that a small number of genes may be missing if their location coincides with the gaps in the CV genome assemblies. We

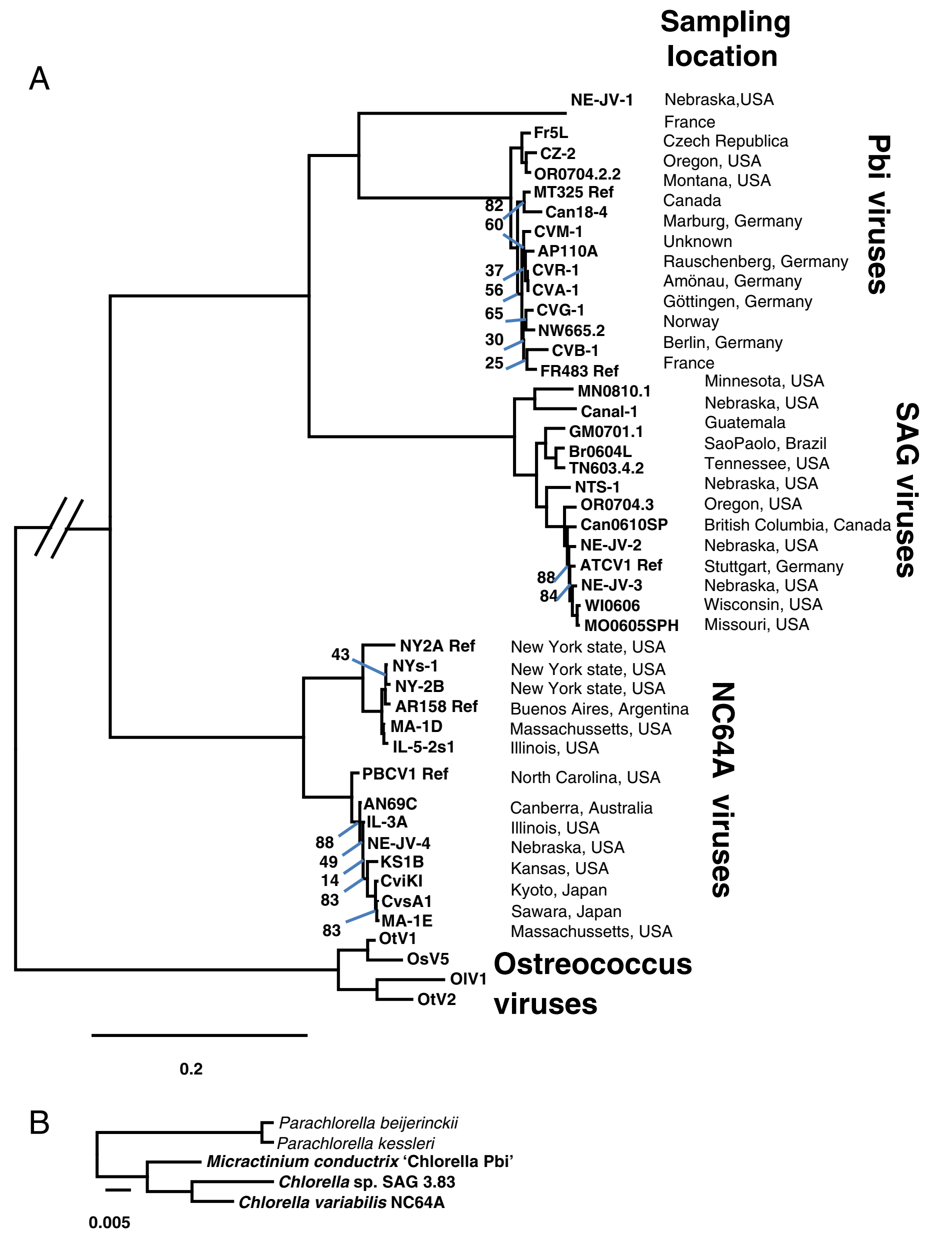

Figure 1 Phylogenies of chloroviruses and algal hosts. A: ML tree of chloroviruses based on a concatenated alignment of 32 core protein families (7762 gap-free sites). The phylogenetic tree was computed using the WAG + G + I substitution model. Branch support was estimated from 1000 bootstrap replicates. We only show bootstrap values < 90%. Ostreococcus viruses serve as an outgroup to root the tree. **B**: ML tree of algal hosts based on 18S RNA alignment (2266 gap-free sites). The phylogenetic tree was computed using the GTR + G + I substitution model. All interior branches received maximal support (100%). *Parachlorella* spp. are used as outgroup.

attempted to complete the assembly of 6 of the incompletely assembled viruses by PCR-sequencing across gaps. However, in many cases, repetitive sequences in adjacent contigs made it difficult to synthesize suitable primers. Since we had >20X depth of coverage in non-repetitive regions, we suspect that the gaps were actually sequenced during the genomic sequencing phase of the project but that the assembly software discarded reads containing repetitive sequences that it was unable to confidently align with sequences at the ends of contigs. Nonetheless, we successfully sequenced 16 of 28 gaps among the 6 viruses and the gap sizes ranged from 1 to 634 nts. Thus the gaps are predicted to be very small.

Core and host-specific proteins in CVs

Predicted CV proteins were organized into 531 clusters of two or more orthologous proteins plus 101 singleton CV proteins (Additional file 1: Table S1). The largest

Table 1 General features of the sequenced *chlorovirus* genomes

Virus	Host	# Contigs	Genome size (Kb)	Sequence coverage	% GC	# protein genes	# tRNA genes	# protein families	Genbank accession number
AN69C	NC64A	8	332	29x	40	362	10	278	JX997153
CviKI	NC64A	8	308	55x	40	336	14	271	JX997162
CvsA1	NC64A	9	310	36x	40	342	14	272	JX997165
IL-3A	NC64A	3	323	50x	40	349	12	273	JX997169
IL-5-2s1	NC64A	9	344	65x	41	379	8	281	JX997170
KS1B	NC64A	7	287	46x	40	319	13	257	JX997171
MA-1D	NC64A	9	339	45x	41	371	11	288	JX997172
MA-1E	NC64A	39	336	27x	40	376	14	269	JX997173
NE-JV-4	NC64A	8	328	41x	40	352	11	276	JX997179
NY-2B	NC64A	5	344	59x	41	371	8	281	JX997182
NYs-1	NC64A	9	348	64x	41	381	7	286	JX997183
AP110A	Pbi	6	327	27x	44	348	9	269	JX997154
Can18-4	Pbi	11	329	52x	45	357	10	271	JX997157
CVA-1	Pbi	8	326	36x	45	346	9	270	JX997159
CVB-1	Pbi	8	319	90x	44	346	10	272	JX997160
CVG-1	Pbi	7	318	48x	45	333	9	262	JX997161
CVM-1	Pbi	5	327	48x	44	341	9	268	JX997163
CVR-1	Pbi	11	329	39x	45	351	9	268	JX997164
CZ-2	Pbi	11	305	39x	45	340	10	262	JX997166
Fr5L	Pbi	22	302	58x	45	345	11	257	JX997167
NE-JV-1	Pbi	8	326	45x	47	337	3	265	JX997176
NW665.2	Pbi	6	325	62x	44	350	8	263	JX997181
OR0704.2.2	Pbi	8	313	53x	45	344	7	261	JX997184
Br0604L	SAG	2	295	65x	49	346	9	272	JX997155
Can0610SP	SAG	1	307	61x	49	341	13	267	JX997156
Canal-1	SAG	4	293	50x	51	336	10	277	JX997158
GM0701.1	SAG	4	315	71x	48	362	10	272	JX997168
MN0810.1	SAG	6	327	57x	52	343	9	268	JX997174
MO0605SPH	SAG	3	289	107x	49	323	11	271	JX997175
NE-JV-2	SAG	4	319	40x	48	346	13	271	JX997177
NE-JV-3	SAG	3	298	63x	49	334	12	268	JX997178
NTS-1	SAG	4	323	35x	48	364	7	271	JX997180
OR0704.3	SAG	5	311	49x	49	342	13	272	JX997185
TN603.4.2	SAG	3	321	28x	49	351	9	276	JX997186
WI0606	SAG	7	289	58x	50	329	11	271	JX997187

protein family contained 429 members, which were similar to intron-encoded endonucleases.

The core protein family set consisted of 155 protein families shared by all the CVs, which represent 56% of the average protein family content of CVs; the majority (66%) of those proteins have an annotated function. Thirty-eight core protein families were also ubiquitous in four *Ostreococcus* viruses [24-27], which are members of the genus *Prasinovirus* that are closely related to the chloroviruses; these core proteins include the NCLDVs hallmark genes (DNA polymerase B, major capsid protein, primase-helicase, packaging ATPase and transcription factor TFII) [2]. The remaining 117 CV core protein families grouped into a variety of functions, with a preponderance of proteins associated with the virion particle (i.e., capsid proteins), degradation of the host cell-wall (i.e., alginate lyase, chitinase and chitosanase), DNA replication, transcription and protein maturation.

These enzymatic functions and structural proteins form the backbone of CV metabolism that enable them to propagate, spread from host to host, enter into the cell, and regulate the cellular machinery to promote virus replication.

In addition, orthologous protein families were identified that are ubiquitous to viruses infecting one of the algal hosts (i.e., NC64A, SAG or Pbi) but absent in all the other CVs. These proteins are presumably involved in the mechanism of host recognition and specificity. The host-specific protein sets were much smaller both in terms of size and number of predicted functions. We identified 11 orthologous clusters specific to NC64A viruses, of which 2 have annotated functions, including an aspartate carbamoyltransferase involved in *de novo* pyrimidine biosynthesis in the plastids of land plants [24], and an homolog to a plant thylakoid formation protein involved in sugar sensing and chloroplast development [25]. This suggests that the adaptation of CVs to the NC64A host might require a more intricate control of the chloroplast and nucleotide biosynthesis by the NC64A viruses. The NC64A viruses have the most biased nucleotide composition of all the CVs (i.e., 40% G + C), which may explain why these viruses require a higher degree of control of the available nucleotide pool. Pbi and SAG viruses had 6 and 9 host -specific core genes, respectively, none of which have known functions, making it difficult to predict the mechanisms underlying host specificity.

Eight protein families had an opposite conservation pattern; they were systematically absent in viruses infecting the same algal host but were present in all the other CVs. Four of them had a predicted function: SAG and NC64A viruses lack an ankyrin repeat domain-containing protein and a glycosyltransferase, respectively. Pbi viruses lack GDP-D-mannose dehydratase and GDP-L-fucose synthase that catalyze two consecutive steps in the biosynthesis of GDP-L-fucose. GDP-L-fucose is the sugar nucleotide intermediate in the synthesis of fucosylated glycolipids, oligosaccharides and glycoproteins [28]. These two enzymes exist in all the other sequenced phycodnaviruses that infect green algae, including *Ostreococcus* viruses, *Micromonas* viruses, and *Bathycoccus* viruses. The long ancestry of GDP-D-mannose dehydratase and GDP-L-fucose synthase suggests that GDP-L-fucose is an important metabolite in the general metabolism of phycodnaviruses that infect green algae. Thus the loss of these two corresponding genes in the Pbi virus lineage may be regarded as a significant evolutionary step that could mark specialization to the host. However, experimental evidence indicates that two sequenced Pbi viruses, MT325 and CVM-1, have fucose as one of the components of their major capsid protein (Tonetti et al., personal communication), indicating that even in the absence of the viral-encoded proteins, Pbi viruses obtain GDP-L-fucose from their host. The loss of the two genes was perhaps made possible by either a greater availability of fucose in the cytoplasm of the Pbi host or a lesser need for fucose by the virus.

The remaining 443 protein clusters had scattered distributions among CVs infecting the three algal hosts. In contrast to the core CV protein set, these protein sets included a significant number of proteins potentially involved in cell-wall glycan metabolism and protein glycosylation, ion channels and transporters, polyamine metabolism, and DNA methytransferases and DNA restriction endonucleases. The different combinations of dispensable genes existing in the CVs are presumably the origin of the phenotypic variations observed between them such as efficiency of infection, burst size, infection dynamics, nature of protein glycans, and genome methylation [11].

Novel protein genes

One hundred and sixty-six clusters totaling 403 proteins did not have an orthologous member in any of the reference viruses. The corresponding genes are thus seen for the first time in CV and encode potential new functionalities. Only 22 new clusters had a match in a public database, the rest of the proteins were annotated as “hypothetical protein.” Furthermore, only 6 clusters were homologous to proteins annotated with functional attributes (Additional file 2: Table S2). They include a fumarate reductase possibly involved in anaerobic mitochondrial respiration [29], and five proteins with generic functional annotation: acetyltransferase, SAM-dependent methyltransferases, nitroreductase, glycosyl hydrolase and helicase.

Phylogeny

Phylogenetic relationships between the sequenced CVs and *Ostreococcus* viruses were determined from an analysis of the concatenated alignment of 32 protein families encoded by a single gene in each genome. *Ostreococcus* viruses were treated as an outgroup to root the phylogenetic trees. These genes represent a subset of the “core” CV genes and are mostly involved in basic replication processes. The resulting maximum likelihood (ML) phylogenetic tree is shown in Figure 1A. All branches are associated with high bootstrap values (>90%) except for those containing very similar viruses, for which the exact timing/order of separation events could not be resolved unambiguously. Phylogenetic trees were also inferred by Neighbor Joining (NJ) and Maximum Parsimony (MP) methods using the same sequence dataset (Additional file 3: Figure S1 and Additional file 4: Figure S2). The MP tree had a topology identical to the ML tree while the NJ tree differed by 5 branches associated with low bootstrap values in both the ML and MP trees. In addition, a ML phylogenetic tree of the algal hosts was reconstructed (Figure 1B) from their 18S RNA sequences using *Parachlorella* species as the outgroup on the basis of a previous phylogenetic study of *Chlorellaceae* [12].

The phylogeny study revealed three important features about CV evolution. First, although the CVs were isolated from diverse locations across 5 continents, the phylogenetic trees show that viruses infecting the same algal host always clustered in monophyletic clades. This suggests that the most recent common ancestor of each virus subgenus already infected the same algal host lineage as today's representatives and that the evolutionary events that led viruses to adapt and specialize to a given host occurred only once in their history. Second, the branching pattern of the three main virus clades does not follow the phylogeny of their algal hosts, which rules out the simplest co-evolution scenario whereby the algae and virus lineages separated in concert. Instead, the phylogenetic evidence strongly suggests that the CVs have changed hosts at least once in their evolutionary history. Finally, while most of the newly sequenced CVs are a close relative of previously sequenced CVs, the basal and isolated phylogenetic position of virus NE-JV -1 within the Pbi virus clade make it the first representative of a new subgroup of CVs that was previously unknown. NE-JV-1 only shares 73.7% amino acid identity on average with the other Pbi viruses in the 32 core proteins used for phylogeny reconstruction. For comparison, the within-clade average protein sequence identity was 92.6%, 95.0% and 97.4% identity for NC64A, SAG and Pbi (excluding NE-JV-1) viruses, respectively. Between clades, the protein sequence identity ranged from 63.1% (NC64A vs. Pbi viruses) to 70.6% (Pbi vs. SAG viruses).

Genome organization and gene colinearity

Figure 2 indicates that gene order is highly conserved among viruses infecting the same algal host, with only a few readily identifiable localized rearrangements, including inversions and indels (see below). Note that the order of contigs in assemblies was determined by maximizing sequence colinearity with the reference genomes. Indeed, 16 gaps were sequenced among six of the new viruses, the primers of which were designed based on the co-linearity of the previously sequenced chloroviruses; however, we cannot eliminate the possibility that additional inversion events exist if their boundaries precisely coincide with the contig ends. The high conservation of gene order contrasts strongly with the low residual gene colinearity between genomes from viruses infecting different algal hosts. The largest conserved genomic regions between CVs infecting different hosts encompassed 32 colinear genes. This observation is consistent with the reported high level of gene colinearity between the genomes of PBCV-1 and NY-2A, two NC64A viruses, as well as between those of MT325 and FR483, two Pbi viruses, but not between NC64A viruses and Pbi viruses [15,17]. We only found one exception to this rule: although the NE-JV-1 virus infects Pbi cells, its gene order is different from that of other Pbi infecting viruses. This lack of gene colinearity is consistent with the basal phylogenetic position of NE-JV-1 within the Pbi virus clade (Figure 1A). NE-JV-1 also has no long-range conserved gene colinearity with NC64A viruses or SAG viruses. This overall lack of colinearity with reference genomes was an issue when ordering the NE-JV -1 contigs between each other using the maximal sequence colinearity criterion. Thus, the order of contigs in the presented NE-JV-1 assembly must be taken with caution. In contrast, although the NC64A viruses also form two separate phylogenetic sub-groups – one sub-group contains PBCV-1 and the other NY-2A – genomes from both sub-groups share an almost perfect gene colinearity as exemplified by the dot-plot comparison between CviKI (PBCV-1 sub-group) and NYs-1 (NY-2A sub-group).

Gene order in *Mimiviridae* genomes is conserved toward the center of the genomes while significant disruptions of gene colinearity occur at the chromosome extremities [30]. This same conservation pattern occurs in *Poxviridae* genomes [31] suggesting that these two families of large DNA viruses, despite their considerable differences, might have evolved under common evolutionary processes linking replication and recombination. In contrast, no obvious differences were observed in the levels of conservation between the center and extremities of the CV genomes, suggesting a different mechanism of genome evolution in this viral clade. The levels of divergence between the colinear genomes of *Mimiviridae* and *Poxviridae* were comparable to the level of divergence between the most distant CV genomes that share no conserved gene colinearity; e.g., DNA polymerase proteins had 64% identical residues between *Mimivirus* and *Megavirus* (*Mimiviridae*) and 65% identical residues between deerpox and variola viruses (*Poxviridae*) [30], while the most divergent CV DNA polymerase protein pair shared 64% identical residues between the SAG virus OR0704.3 and NC64A virus MA-1D. Taken together, these observations suggest that at comparable genetic distances, genome rearrangements were more frequent in CVs than in *Mimiviridae* and *Poxviridae*.

Some spontaneous antigenic variants of PBCV-1 contained 27- to 37-kb deletions in the left end of the 330-kb genome [32]. Although these mutant viruses stably replicate in the *C. variabilis* host in laboratory conditions, albeit with phenotypic variations compared to the PBCV-1 wild type strain, it was unknown if such

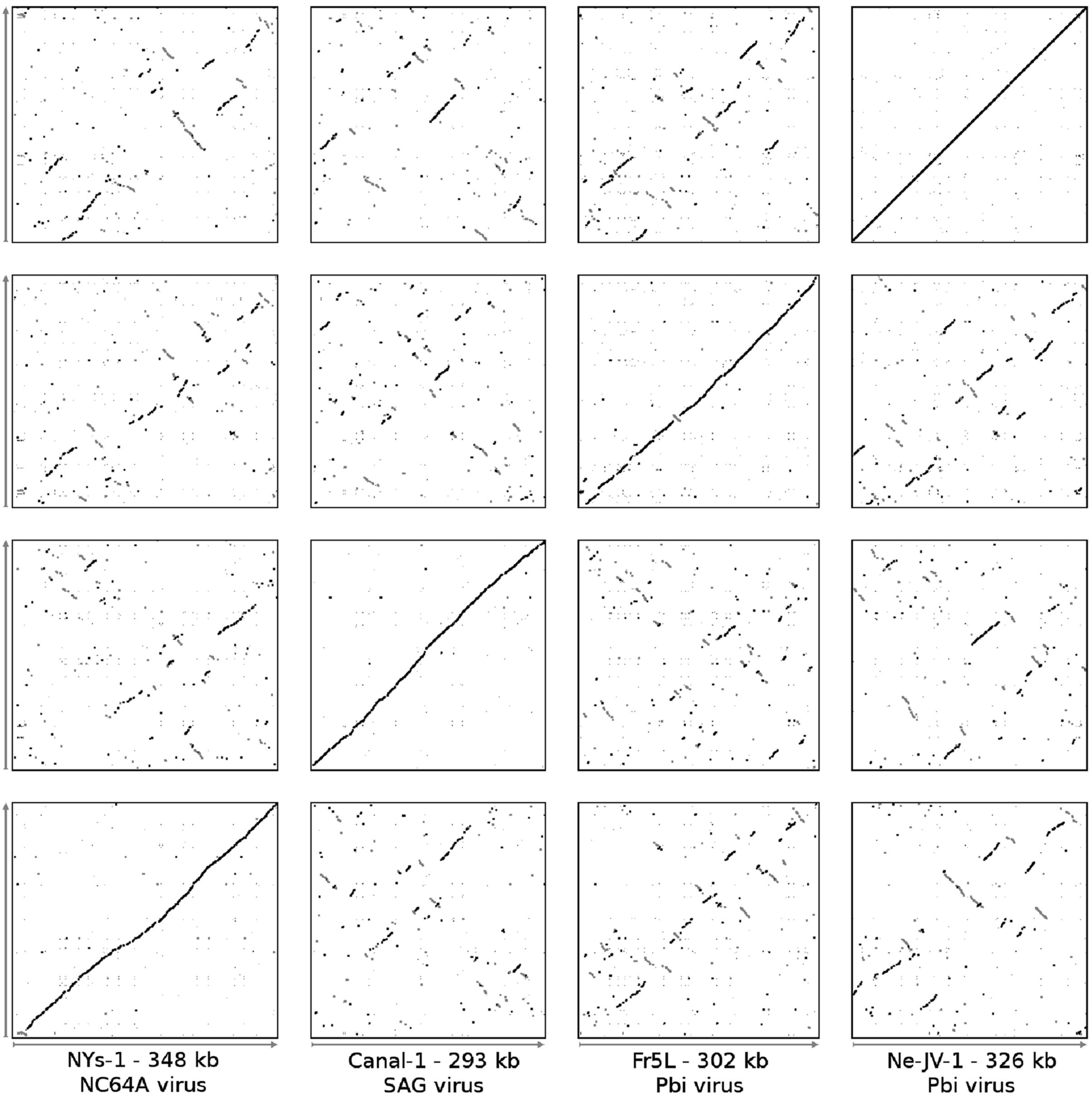

Figure 2 Dot-plot alignments of ten newly sequenced *Chlorovirus* genomes. Each dot represents a protein match between two viruses (BLASTP e-value < 1e-5) from genes in the same orientation (black) or in reverse orientation (gray). Best BLAST matches are shown with larger dots.

mutants existed in natural populations. The NC64A virus KS1B isolated in Kansas, USA contained a 35-kb deletion in the left end, when compared to the PBCV-1 wild type. This finding suggests that the deleted region that encompasses 29 ORFs in the PBCV-1 genome is dispensable in a natural environment. The missing PBCV-1 ORFs encode 2 capsid proteins, a pyrimidine dimer-specific glycosylase and 26 putative proteins with unknown function (Additional file 5: Table S3). Thus the KS1B virus may have altered capsid and DNA repair capability. Further study is required to determine if the KS1B genotype is common and stably fixed in the natural population or if it is a rare mutant that was sampled by chance or if it results from a recent mutation that occurred during maintenance of the virus in the laboratory.

Origin of the CV genes

Reconstruction of ancestral genomes using the maximum parsimony method predicts that the last common ancestor of all sequenced CVs encoded at least 297 protein families (Figure 3A), including 155 core CV protein

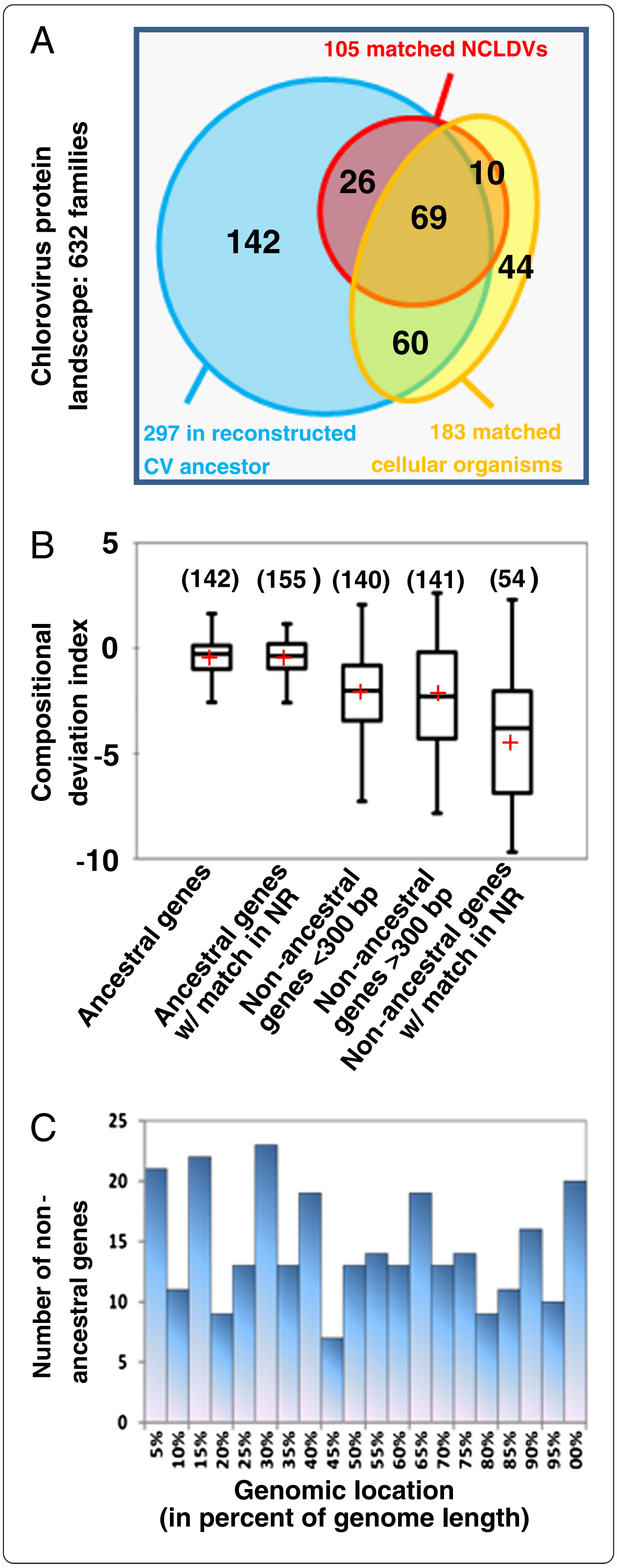

Figure 3 Characteristics of *Chlorovirus* protein families. A: Distribution of protein families in the ancestral and non-ancestral subsets. **B**: Box-plot distributions of median compositional deviation index (CDI) in gene families. The number of gene families in a category is given in parentheses. Distribution means are shown by a red cross; medians are shown by horizontal lines in boxes. **C**: Distribution of genomic locations of non-ancestral gene families. For each family, we recorded the average genomic location for gene members that occur in colinear genomes.

families plus 142 families that were lost in one or more modern CV genomes. This result suggests that the last common CV ancestor had a gene pool size slightly bigger than the extant viruses that encode 257 to 288 protein families (Table 1). The ancestral families account for 82% to 88% of the protein repertoire in the modern CVs. One hundred and five ancestral CV proteins also had homologs in other NCLDV genomes and were potentially inherited from an even older NCLDV ancestor; however, 335 (53%) of the 632 predicted chlorovirus protein families could not be traced back to the CV ancestor, which most probably also infected chlorella-like hosts. A fraction of them were presumably encoded in the ancestral genome and subsequently lost in all of the NC64A, Pbi and SAG viruses, so that their occurrence in the common ancestor could not be established using the parsimony criterion. Furthermore, we cannot rule out that some of the ORFan genes (ORF without match in sequence databases and the other chlorovirus subgenera) are erroneous predictions. Sequence randomization between non-ORFan genes indicates that on average less than 1 ORF >300 bp in size can be obtained by chance in a chlorovirus genome; 185 non-ancestral protein families were encoded by ORFs that have a median length >300 bp. Alternatively, the corresponding genes could have been gained after the divergence of the main CV clades. There are three known mechanisms that can lead to gene gain: duplication of existing genes, capture of genes from other genomes through horizontal gene transfer (HGT) and creation of new genes from noncoding sequences *de novo*. Although gene duplicates exist in the CVs, they were not considered in subsequent analyses because in-paralogs were aggregated into existing orthologous clusters in the construction of the protein families.

Non-ancestral genes

The oligonucleotide frequency in a sequence is known to be species-specific and can be used as a genomic signature [33]. Since DNA transfers originate from species with a compositional signature different from that of the recipient species, significant deviation of a signature between ORFs and the rest of the genome may signal recently transferred DNA. For each virus we constructed a five-order non-homogeneous Markov chain model of nucleotide frequency in the ORFs that were identified as being vertically inherited from the last common CV ancestor (i.e., ancestral ORFs). This model was used to compute a compositional deviation index (CDI) for ancestral and non-ancestral ORFs. The distributions of CDI values shown in Figure 3B differed significantly between ancestral and non-ancestral ORFs (Kruskal–Wallis test p < 0.0001 and Steel-Dwass-Critchlow-Fligner W* test p < 0.0001 between each pairwise combination of ancestral and non-ancestral CDI subsets). On average, non-ancestral ORFs had lower CDI values meaning that their nucleotide composition tends to exhibit a poorer fit to the nucleotide frequency model. This trend was true irrespective of the identification of homologs in databases or not. Furthermore, the distributions of CDI values for long (>300 bp) and short (<300 bp) ORFan families were not significantly different (Mann–Whitney test p ~0.99). This suggests that at least a fraction of the non-ancestral genes, including the genes with no recognizable homologs in the database, have been captured by HGT from genomes with distinct nucleotide compositional biases and that this event was sufficiently recent so that the difference in nucleotide composition is still visible.

To test this hypothesis, phylogenetic trees were reconstructed from 35 of the 54 non-ancestral protein families that had significant matches in Genbank. For the remaining 19 protein families, no reliable phylogenetic tree could be generated due to the scarcity of homologous sequences or too high sequence divergences between homologs. Most of the 35 phylogenetic trees were not conclusive as to the exact evolutionary history of the viral genes (Phylogenetic trees are shown in Additional file 6: Figure S3 and a summary of the interpretations is shown in Additional file 7: Table S4): In many cases, CV proteins had relatively deep branches in the tree implying that if the hypothesis of a recent HGT is supportable, sequences of the donor genome or its close relatives are not available in databases. Moreover, cellular homologs were sometimes sporadically distributed among eukaryotes, bacteria and sometimes viruses, and phylogenetic trees exhibited major discrepancies with the accepted phylogeny of the organism. Altogether these results suggest that these proteins are encoded by genes that were frequently exchanged between cellular organisms and between cellular organisms and viruses. In nine of the phylogenetic trees the CV proteins branched as a sister group to green algae or land plants. However, in only one case did the CV proteins directly branch on the *C. variabilis* branch, i.e., a tree topology consistent with a recent HGT between viruses and hosts. This HGT was readily identified as a capture of the algal dUDP-D-glucose 4,6-dehydratase gene by SAG viruses because the viral protein clade branched within the green algal phylogenetic sub-tree (CL0780 in Additional file 6: Figure S3). Thus, except for this obvious case, the origin of the green algal-like viral genes is unclear. Three alternative scenarios can explain this incongruence: (i) CVs captured green algal genes during infection of other algae that are distantly related to these hosts. However, this hypothesis is not consistent with the apparent specificity of CVs for one of the three algal strains. (ii) CVs captured genes from their “natural” algal host(s) but these genes have been lost in the genome of the model strain *C. variabilis* NC64A. (iii) CVs captured genes within the algal host from other parasites or symbionts (viruses or bacteria) that contain green algal genes. In fact, 18 phylogenetic trees placed CV proteins in a sister position to bacteria. For six of the concerned protein families, homologs were also found in phages or other DNA viruses.

Thus, although the non-ancestral genes exhibit specific compositional features suggesting this subset is enriched in sequences with a potential extraneous origin, a majority of them (281 families) have no identifiable homolog in the databases, and for those that do (54 families), only a few produced a phylogenetic tree where the clade of the donor organism could be identified with a reasonable degree of confidence. Thus, if the hypothesis of acquisition by HGT is supported for the non-ancestral CV genes, they must originate from a DNA fraction that is under-represented in public databases.

Finally, we investigated the location of the non-ancestral genes within the CV genomes. The non-ancestral genes are evenly distributed across the CV genomes (Figure 3C). This contrasts with the cases of *Mimiviridae* and *Poxviridae*, which have genus- and species-specific genes clustered toward the extremities of their genomes, whereas the conserved genes are clustered in the middle [30,34]. This result reinforces the apparent differences between the evolution of CV genomes and that of the members of other NCLDV clades.

Gene exchanges with the algal host

Previous studies attempted to identify genes of cellular origin in CV genomes [35]. It was estimated that 4 to 7% of CV genes are of bacterial origin, and an additional 1 to 2% were acquired from the plant lineage [36] though interpretation of the results was subject to controversy [37]. These low numbers put into question the real significance of HGT in CVs; however, the genome of the host for the NC64A viruses was not sequenced at the time of these previous studies. Since the release of the *C. variabilis* genome sequence [38], no systematic study of gene exchanges between CVs and the algal host has been undertaken. It should be noted that the SAG virus host, *C. heliozoae*, and Pbi host, *M. conductrix*, have not been sequenced. However, their close phylogenetic relationships with the host for the NC64A viruses permit using the *C. variabilis* genome as a proxy for the other host species. The above analysis of the non-ancestral protein families already identified a case of gene acquisition by SAG viruses from the host; we completed this study by investigating the phylogenetic affinities in the ancestral protein family subset.

Out of the 297 ancestral families, 42 had significant matches with *C. variabilis* homologs. Subsequent phylogenetic analysis identified seven families where the viral protein clades branched next to *C. variabilis* homologs, reflecting potential HGT between viruses and the host (Additional file 8: Figure S4). For two of them, the likely direction of HGT could be inferred as a capture of the algal gene by the CV ancestor based on the placement of the CV branch within the green algae clade. These 2 genes encode a translation elongation factor EF-3 (CL0450) and an unknown protein (CL0511). In yeast, EF-3 interacts with both ribosomal subunits and facilitates elongation factor EF-1-mediated cognate aminoacyl-tRNA binding to the ribosomal A-site [39]. Thus, capture of the algal EF-3 gene may help CVs by enhancing protein biosynthesis during infection. For the 4 remaining families (chitin deacetylase, chitinase and 2 unknown proteins), *C. variabilis* is the only plant organism to share these viral genes; thus their vertical inheritance from an ancestor is more unlikely as this would imply many subsequent gene losses among the other descendants of the plant ancestor. An alternative scenario involves gene captures by the algal host from the virus genome. Although no lysogenic cycle has yet been identified among CVs, some members of the phycodnavirus family are known to integrate into the host genome [40]. Thus, these algal genes may correspond to remnants of ancient integrated genomes of unknown lysogenic viruses.

Altogether, these results suggest that the CVs and their hosts did not frequently exchange genes. Overall, only 3 genes showed evidence of capture through host-to-virus exchanges and in 4 other genes the opposite scenario is more likely (virus-to-host exchange). Furthermore, 2 of the host-to-virus exchanges occurred before the divergence of the CVs (i.e., in ancestral protein families), suggesting that they could have contributed to the early adaptation of viruses to their algal host. Thus, although large viruses are often presented as mainly evolving by recruiting genes from their hosts, this conjecture does not hold true for the CVs.

Conclusion

One of the most striking findings from this study is that more than half of the CV predicted protein families are encoded by genes of recent extrinsic origin (after Chlorovirus divergence) – most of which are also ORFans. The proportion of non-ancestral genes in individual CV genomes is substantial–12% to 18% of the protein families–though this proportion is similar to atypical genes of likely extrinsic origin in bacterial genomes [38]; however clues as to the potential donor genomes are lacking. The algal host cytoplasm is probably the sole milieu where the viral genome is accessible for recombination and acquisition of extrinsic genes. Consequently horizontally transferred genes can arise from 3 potential sources: (i) host DNA, (ii) bacterial DNA, and (iii) DNA from other (perhaps distantly related) viruses competing for the same algal host.

Our study shows that the capture (and fixation) of algal host DNA has been rare in the evolutionary history of CVs and cannot explain the vast majority of non-ancestral CV genes. Furthermore, we believe that bacterial DNA is not a major source of extrinsic genes in CVs: if non-ancestral genes were mainly of bacterial origin we would expect that the proportion of ORFans in the non-ancestral gene data set to be comparable to the proportion of ORFans in bacterial genomes. Estimated frequencies for ORFans in bacterial genomes vary between 7% for the most recent estimates [41] to 20–30% for estimates made early in the history of genome analysis [42], when only the first organisms had been sequenced. These frequencies are significantly below the frequency of ORFans in the non-ancestral CV protein family dataset (from 141/195 = 72% if we only consider "long" ORFans to 281/335 = 84% if we consider all predicted genes).

Thus if the conjecture of acquisition by HGT is true for the non-ancestral CV genes, they must originate from a still largely un-sequenced reservoir of genomes. The biological entities that match best with this characteristic are the viruses themselves. Viruses are by far the most abundant entities in aquatic environments and we are only now realizing the extraordinary range of global viral biodiversity [8]. Thus, we suspect that the apparent incongruence between compositional evidence of HGT and lack of donor (or close relative) sequences in the databases reflect the fact that non-ancestral CV genes arose from recombination with other unknown viruses that infect the same hosts. However, this does not rule out alternate hosts that could be underrepresented in the existing databases as possible donors.

Methods

Virus isolation and storage

The set of viruses used in this study were collected at different times over several years from various terrestrial waters around the world (see Additional file 9: Table S5). The water samples were evaluated for plaque-forming viruses on the specific algal host, and the plaque isolates were chosen based on phenotypic characteristics of interest or for geographic distribution purposes. The intention was to evaluate a broad spectrum of chloroviruses with approximately an equal number infecting each of the three algal hosts. The plaque isolates were plaque purified at least two times, then amplified in liquid culture for the purposes of virus purification using the method previously described [14]. The purified viruses were plaque assayed to determine the number of infectious particles and stored at 4°C.

DNA isolation

Viral DNA was purified from virions that had been treated with DNase I (10 units/ml in 50 mM Tris–HCl pH 7.8/1 mM $CaCl_2$/10 mM $MnCl_2$ at 37°C for 1 hr), using the UltraClean®Blood DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The DNA was evaluated for quantity and quality by measuring absorbance at 260 and 280 nm with a Thermo Scientific NanoDrop 2000 spectrophotometer, and by measuring fluorescence of dye-augmented DNA using the PicoGreen and a Qubit fluorometer (Invitrogen, Carlsbad, California).

Genomic library preparation and sequencing

Genomic libraries were constructed from pairs or triplets of pooled viral genomic DNA. A schematic representation of the multiplexed sequencing pipeline is shown in Additional file 10: Figure S5. Using the Roche Rapid Library Preparation method for GS FLX Titanium chemistry (Roche 454 Life Sciences, Branford, Connecticut), sample DNA was fragmented by nebulization. DNA fragments were end repaired with polynucleotide kinase and T4 DNA polymerase, then purified by size exclusion chromatography. Selected DNA fragments were ligated to a Rapid Library Multiplex Identifier (MID) adaptor designed for GS FLX Titanium chemistry. The MID adaptors were designed with a unique decamer sequence to facilitate multiplex sequencing with the 454 technology, such that the resulting library reads can be reliably sorted after sequencing using SFF software tools. MID adaptor ligated DNA fragments were again size selected by chromatography, quantified with a TBS-380 mini-fluorometer (Promega, Madison, Wisconsin). The Rapid Library quality was assessed with an Agilent Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, Santa Clara, California). The average fragment length was between 600 bp and 900 bp, with the lower size cut-off at less than 10% below 350 bp. Pooled DNAs were titrated to obtain the optimal copies per bead (cpb). After titration, 3 cpb was chosen as the best DNA and bead ratio and corresponding amounts of DNA were added to the subsequent emPCR reactions. EmPCR was performed with the 454/Roche Lib-L (LV) kits following manufacturer's protocol for the Roche 454 GS FLX Titanium.

Sequence assembly and gene prediction and annotation

All of the viral DNA genomic libraries, as emPCR products, were sequenced through two duplicated multiplex runs on a Roche GS FLX Titanium sequencer. 454 image and signal processing software v.2.3 generated a total of 2,434,736 PassedFilter reads after removing reads under 40 bp in length. The raw data from the 454-pyrosequencing machine were first processed through a quality filter and only saved sequences that met the following criteria: i) contained a complete forward primer and barcode, ii) contained no more than one "N" in a sequence read where N is equivalent to an interrupted and resumed signal from sequential flows, iii) reads were 200 to 500 nts in length, and iv) reads had a average quality score of 20. Using SFF tools implemented in the 454 GS-Assembler 2.3, each read was trimmed to remove 3' adapter and primer sequences and was parsed by a MID adaptor barcode. The corresponding QUAL file also was updated to remove quality scores from reads not passing quality filters. This procedure allowed the unambiguous assignment of 2,429,860 reads of 384-bp on average to the corresponding genomic libraries

Separate assembly for each library was performed by the MIRA assembler version 3.2.0 using the following parameters: --job = denovo, genome, accurate, 454 -DP: ure = yes -CL:emrc = yes -AL:mo = 50 -ED:ace = yes. Overall a total of 1557 contigs containing 2,330,493 reads were generated.

The resulting contigs were assigned to their corresponding viruses and ordered between each other by alignment against reference viral genomes, e.g. PBCV-1, NY-2A, and AR158 for NC64A viruses [GenBank: JF411744, DQ491002, DQ491003], ATCV-1 for SAG viruses [GenBank:EF101928] and MT325 and FR483 for Pbi viruses [GenBank:DQ491001, DQ890022].

A first list of putative ORFs was constructed using the GeneMarkS program (using the -lo and -op options) [43]. A list of potential ORFs (size >60 codons) occurring in the intergenic regions between GeneMarkS predicted genes was compiled. These potential ORFs were added to the predicted gene list only if they had a significant match (BLASTP e-value < 1e-5) in the Genbank non-redundant (nr) database, omitting matches in the *Chlorovirus* genus. Predicted proteins were functionally annotated based on match against multiple sequence databases, including Swissprot, COG, Pfam and nr databases using an e-value threshold of 1e-5 for both BLASTp and HMMer searches. tRNAs genes were predicted using the tRNAscan-SE program, ignoring pseudo- and undetermined-tRNAs.

Protein clustering

Putative orthologous protein pairs were first identified using the reciprocal best BLASTp hit criterion and assembled into orthologous clusters by the single-linkage clustering method. Putative orthologous proteins of four sequenced *Ostreococcus* viruses were also included in the clustering scheme to serve as an outgroup in subsequent analyses. In-paralogs (resulting from the duplication of a protein gene after divergence of two viral lineages) were assigned to existing orthologous clusters if their alignment scores with one protein of a cluster were greater than any of the alignment scores between this protein and the other members of the cluster.

Phylogenetic analysis

Phylogenetic analysis was performed using the following general pipeline: homologous sequences were searched in databases using the BLAST EXPLORER tool [43]. Multiple-sequence alignments were performed using the MUSCLE program [44], followed by manual edition, and removal of gaped sites and poorly aligned regions. Phylogenetic trees were reconstructed using the PHYML program (Maximum likelihood) [45] and Mega 4 (Neighbor Joining and maximum parsimony) [46]. Statistical support for branches was assessed using 1000 bootstrap datasets.

Chlorovirus ancestor gene content

Given the phylogeny of the sequenced CV shown in Figure 1A, protein families that contained at least one member in one of the NC64A viruses and at least one member in one of the Pbi viruses or SAG viruses were considered as being inherited from the last common CV ancestor. A total of 290 protein families were identified as “ancestral” by this procedure. In addition, 7 protein families that are a sister group to homologs in NCLDV in phylogenetic ML trees were considered to be inherited from the last common ancestor. Thus the genome of the last common CV ancestor was inferred to encode at least 297 protein families.

Compositional deviation index

To distinguish between intrinsic and extrinsic genes, a compositional deviation index (CDI) was computed. The CDI score reflects how much the nucleotide composition of an ORF deviates from that of a reference set of ancestral ORFs. Thus, an extrinsic ORF integrated into the genome is distinguished from the recipient genome sequences by the nucleotide composition, unless the donor and recipient species are close relatives with similar nucleotide compositional biases. Ancient transferred genes may be indistinguishable, because the nucleotide composition of horizontally transferred genes generally converges with that of the recipient genome by mutation pressure. Thus, this procedure preferentially detects recent horizontally transferred genes for which the compositional convergence process has not been completed.

Our method for computing CDI scores was largely inspired from earlier works on gene finding [47] and extrinsic DNA identification [48]; these two references contain detailed explanations of the statistical framework and construction of the model. A non-homogenous Markov model for ancestral coding nucleotide sequences was defined by four components: P^0, the initial probability vector for starting k-bp tuples j in ancestral ORFs, and P^1, P^2, P^3, three transition matrices that define the probability that a k-tuple j whose first nucleotide occupies respectively the f = 1^{st}, 2^{d} or 3^{th} position in a codon, is followed by one of the four possible nucleotides (i). The likelihood of finding an ORF of length l given the model is:

$$P(\mathrm{ORF}|\mathrm{COD_{anc}}) = P^0(j_1)P^1(i_{k+1}|j_1)P^2(i_{k+2}|j_2)P^3(i_{k+3}|j_3)\ldots P^f(i_l|j_{l-k})$$

Numerical values of the parameters of the model (P^0, P^1, P^2 and P^3) were derived from the count of k-tuples N_j and (k + 1)-tuples $N_{(j,i)}$ in the training sequence set containing all ancestral ORF of a CV. That is, initiation probabilities were taken as the frequencies of k-bp tuples, and transition probabilities were equal to $N^f_{(j,i)}/N^f_{(j)}$. The order of the Markov chains was set to five (k = 5) to avoid an overfitting of the parameters.

For each ORF, the CDI value was computed as follows: first the mean and standard deviation (SD) of $P(\mathrm{ORF_r}|\mathrm{COD_{anc}})$ for 100 random coding sequences emitted from the Markov chain model was determined. The random sequences had the same length that the ORF for which CDI was computed. The CDI was calculated according to the formula:

$$\mathrm{CDI_{ORF}} = \mathrm{P}(\frac{\mathrm{ORF}|\mathrm{COD_{anc}}) - \bar{P}(\mathrm{ORF}_r|\mathrm{COD_{anc}})}{\mathrm{SD}_{P(\mathrm{ORF}_r|\mathrm{COD_{anc}})}}$$

The expectation is CDI = 0 for ORFs with nucleotide compositions that fit with the model for ancestral coding nucleotide sequences, while ORFs whose nucleotide composition significantly deviates from the model shall have CDI ≠ 0.

Additional files

Additional file 1: Table S1. 632 *Chlorovirus* protein families.

Additional file 2: Table S2. Example of orthologous protein clusters viewed for the first time in Chloroviruses.

Additional file 3: Figure S1. Neighbor joining tree of the reference concatenated alignment. The NJ tree of chloroviruses is based on a concatenated alignment of 32 core protein families (7762 gap-free sites). Phylogenetic distances were computed using the WAG + G + I substitution model. Branch support was estimated from 1000 bootstrap replicates. We only show bootstrap values < 90%. Branches that differed from the ML and MP trees are colored in red.

Additional file 4: Figure S2. Maximum parsimony tree of the reference concatenated alignment. The MP tree of chloroviruses is based on a concatenated alignment of 32 core protein families (7762 gap-free sites).

Phylogenetic tree was computed using the close-neighbor-interchange method. Branch support was estimated from 1000 bootstrap replicates. We only show bootstrap values <90%.

Additional file 5: Table S3. PBCV-1 genes missing in the KS1B genome as the result of a 35Kb deletion.

Additional file 6: Figure S3. 35 phylogenetic trees of non-ancestral *Chlorovirus* protein families. Trees were reconstructed using the ML method using the WAG + G + I substitution model. Interior branch support was estimated by the approximate likelihood ratio test (aLRT). For the sake of clarity, we only show branch support for important clades. Taxon names are colorized according to taxonomic information: *Chlorovirus* (red), chlorophytes (dark green), streptophytes (light green), eukaryote (violet), prokaryote (pink) and DNA virus (blue). Genbank gi numbers are given after species names. Protein family ID and functional annotation are given above each tree.)

Additional file 7: Table S4. Sister groups to non-ancestral *Chlorovirus* proteins based on 35 phylogenetic trees shown in Additional file 7: Figure S3.

Additional file 8: Figure S4. Phylogenetic trees showing potential HGT between chloroviruses and *Chlorella*. Trees were reconstructed using the ML method using the WAG + G + I substitution model. Interior branch support was estimated by the approximate likelihood ratio test (aLRT). For the sake of clarity, we only show branch support for important clades. Taxon names are colorized according to taxonomic information: *Chlorovirus* (red), chlorophytes (dark green), streptophytes (light green), eukaryote (violet), prokaryote (pink) and DNA virus (blue). Genbank gi numbers are given after species names. Protein family ID and functional annotation are given above each tree.

Additional file 9: Table S5. Attributes of the sequenced chloroviruses.

Additional file 10: Figure S5. Schema of the multiplexed sequencing strategy.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJ: analyzed the data and drafted the manuscript. DDD: Conceived the project, provided several viruses, purified the viruses and the viral DNAs, developed the sequencing libraries, contributed to the sequence assembly and contig assignment, contributed to the data analysis and interpretation and writing of the manuscript. JRG, IA, MK, JV, ML provided essential materials for virus isolation and production. GD, OWM provided essential computational support for virus sequence analyses. JMC: helped to draft the manuscript. JLVE: initiated the project and helped write the final paper. GB: coordinated analysis of the data and drafted the manuscript. All authors read and approved the final manuscript.

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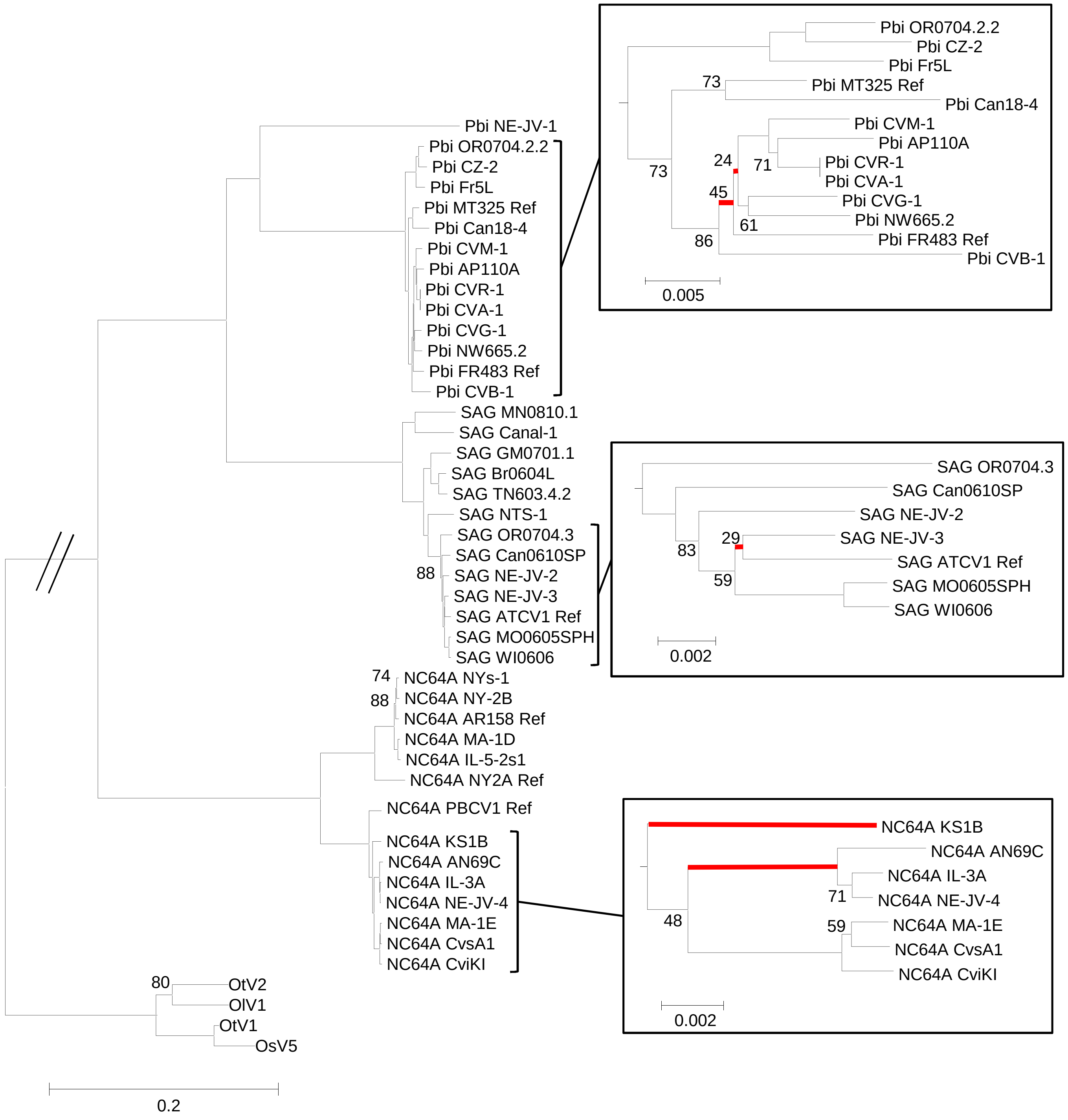
Cite this article as: Jeanniard *et al.*: Towards defining the chloroviruses: a genomic journey through a genus of large DNA viruses. *BMC Genomics* 2013 **14**:158.

Table S2: Examples of orthologous protein clusters viewed for the first time in Chloroviruses.

Cluster ID	Putative function
CL0049	Fumarate reductase
CL0462	Acetyltransferase
CL0875	SAM-dependent Methyltransferase
CL0940	Nitroreductase
CL0963	Glycosyl hydrolase
CL1018	Helicase

Figure S1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Pbi CVA-1
Pbi CVG-1
Pbi NW665.2
Pbi FR483 Ref
Pbi CVB-1
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SAG Canal-1
SAG GM0701.1
SAG Br0604L
SAG TN603.4.2
SAG NTS-1
SAG OR0704.3
SAG Can0610SP
88
SAG NE-JV-2
SAG NE-JV-3
SAG ATCV1 Ref
SAG MO0605SPH
SAG WI0606
74
NC64A NYs-1
88
NC64A NY-2B
NC64A AR158 Ref
NC64A MA-1D
NC64A IL-5-2s1
NC64A NY2A Ref
NC64A PBCV1 Ref
NC64A KS1B
NC64A AN69C
NC64A IL-3A
NC64A NE-JV-4
NC64A MA-1E
NC64A CvsA1
NC64A CviKI
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OtV2
OlV1
OtV1
OsV5
0.2
Pbi OR0704.2.2
Pbi CZ-2
Pbi Fr5L
73
Pbi MT325 Ref
Pbi Can18-4
Pbi CVM-1
Pbi AP110A
24
71
Pbi CVR-1
73
Pbi CVA-1
45
Pbi CVG-1
61
Pbi NW665.2
86
Pbi FR483 Ref
Pbi CVB-1
0.005
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29
SAG NE-JV-3
83
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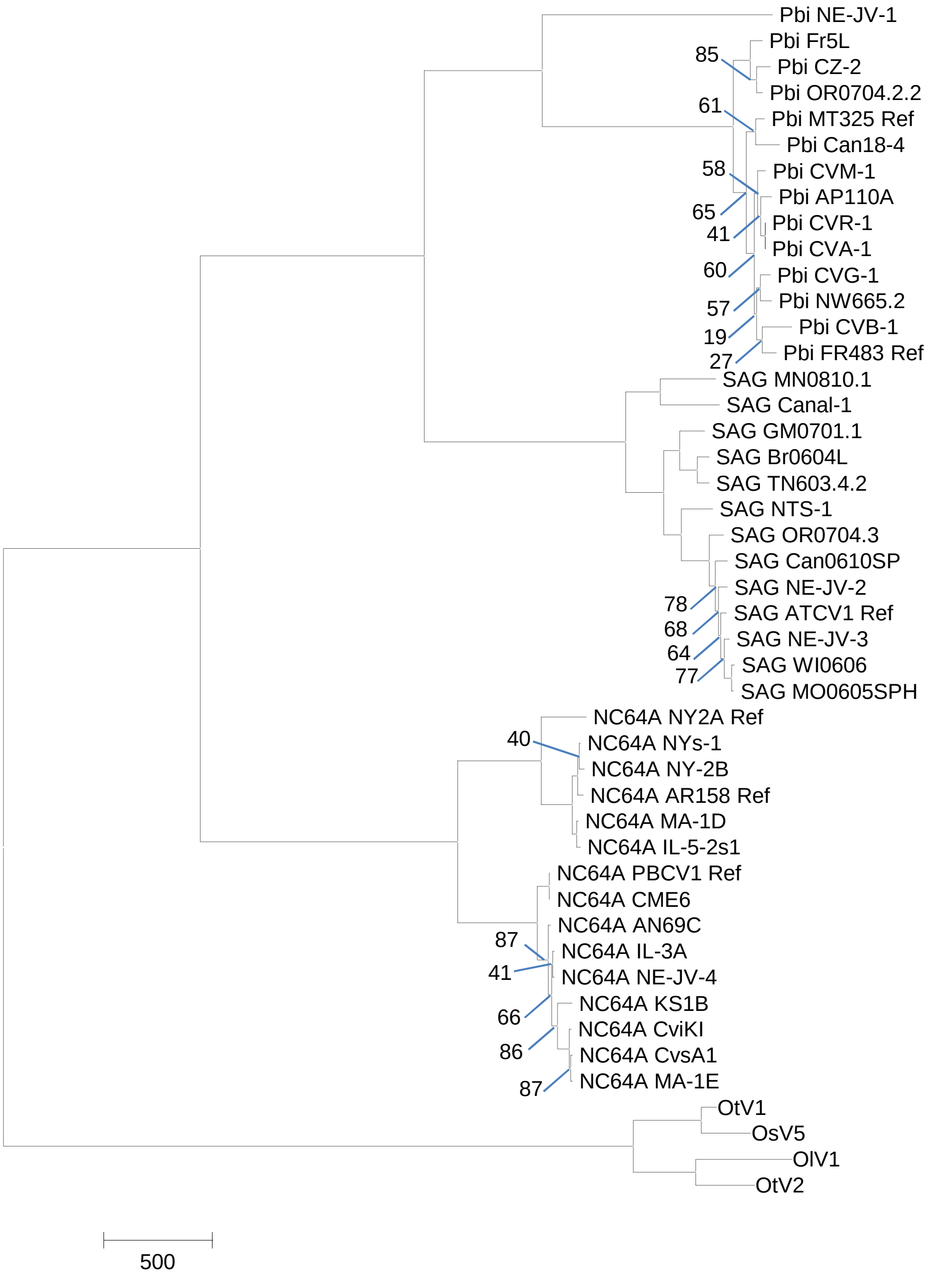
Figure S2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Pbi CVA-1
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Pbi CVG-1
Pbi NW665.2
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Pbi CVB-1
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SAG Canal-1
SAG GM0701.1
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SAG TN603.4.2
SAG NTS-1
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64
SAG WI0606
77
SAG MO0605SPH
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NC64A NYs-1
NC64A NY-2B
NC64A AR158 Ref
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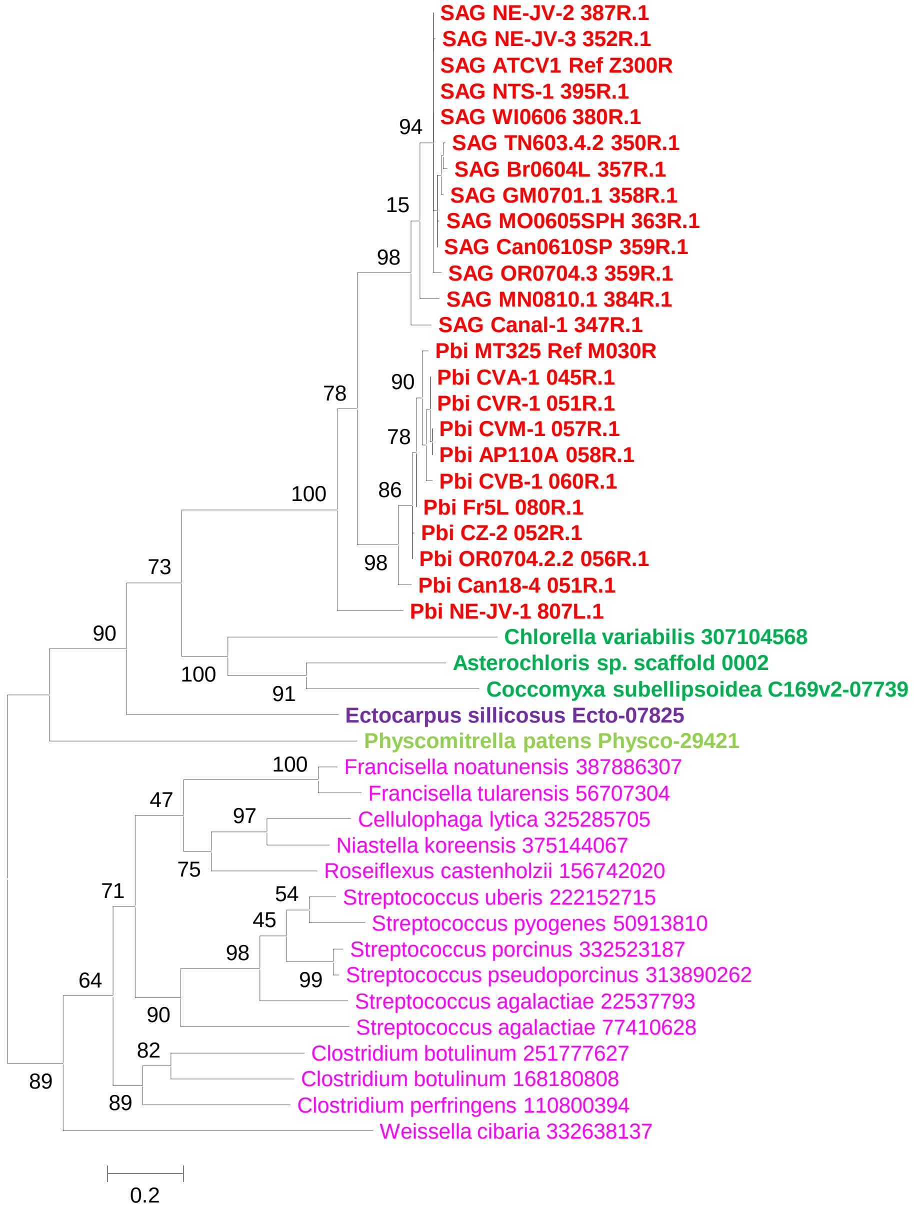
NC64A IL-5-2s1
NC64A PBCV1 Ref
NC64A CME6
NC64A AN69C
87
NC64A IL-3A
41
NC64A NE-JV-4
NC64A KS1B
66
NC64A CviKI
86
NC64A CvsA1
87
NC64A MA-1E
OtV1
OsV5
OlV1
OtV2
500

Table S3 : PBCV-1 genes missing in the KS1B genome as the result of a 35Kb deletion

PBCV-1 gene name	Predicted function
A002L	Unknown protein
A002bL	Hypothetical protein
A002cR	Hypothetical protein
A003R	Unknown protein
A005R	Unknown protein
A007/008L	Unknown protein
A009R	Unknown protein
A010R	Capsid protein
A011L	Capsid protein
A014R	Unknown protein
A018L	Unknown protein
A025/027/029L	Unknown protein
A034R	Protein kinase
A035L	Unknown protein
A037L	Unknown protein
A039L	Unknown protein
A041R	Unknown protein
A044L	Unknown protein
A048R	Unknown protein
A049L	Unknown protein
A050L	Pyrimidine dimer-specific glycosylase
A050aL	Hypothetical protein
A051L	Unknown protein
A053R	Unknown protein
A058L	Hypothetical protein
A057aR	Hypothetical protein
A060L	Unknown protein
A061L	Unknown protein
A063L	Unknown protein

Figure S3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SAG Br0604L 357R.1
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SAG Canal-1 347R.1
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Pbi CVM-1 057R.1
Pbi AP110A 058R.1
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Pbi CZ-2 052R.1
Pbi OR0704.2.2 056R.1
Pbi Can18-4 051R.1
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Asterochloris sp. scaffold 0002
Coccomyxa subellipsoidea C169v2-07739
Ectocarpus sillicosus Ecto-07825
Physcomitrella patens Physco-29421
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Francisella tularensis 56707304
Cellulophaga lytica 325285705
Niastella koreensis 375144067
Roseiflexus castenholzii 156742020
Streptococcus uberis 222152715
Streptococcus pyogenes 50913810
Streptococcus porcinus 332523187
Streptococcus pseudoporcinus 313890262
Streptococcus agalactiae 22537793
Streptococcus agalactiae 77410628
Clostridium botulinum 251777627
Clostridium botulinum 168180808
Clostridium perfringens 110800394
Weissella cibaria 332638137
94
15
98
90
78
78
86
98
100
73
90
100
91
100
47
97
75
71
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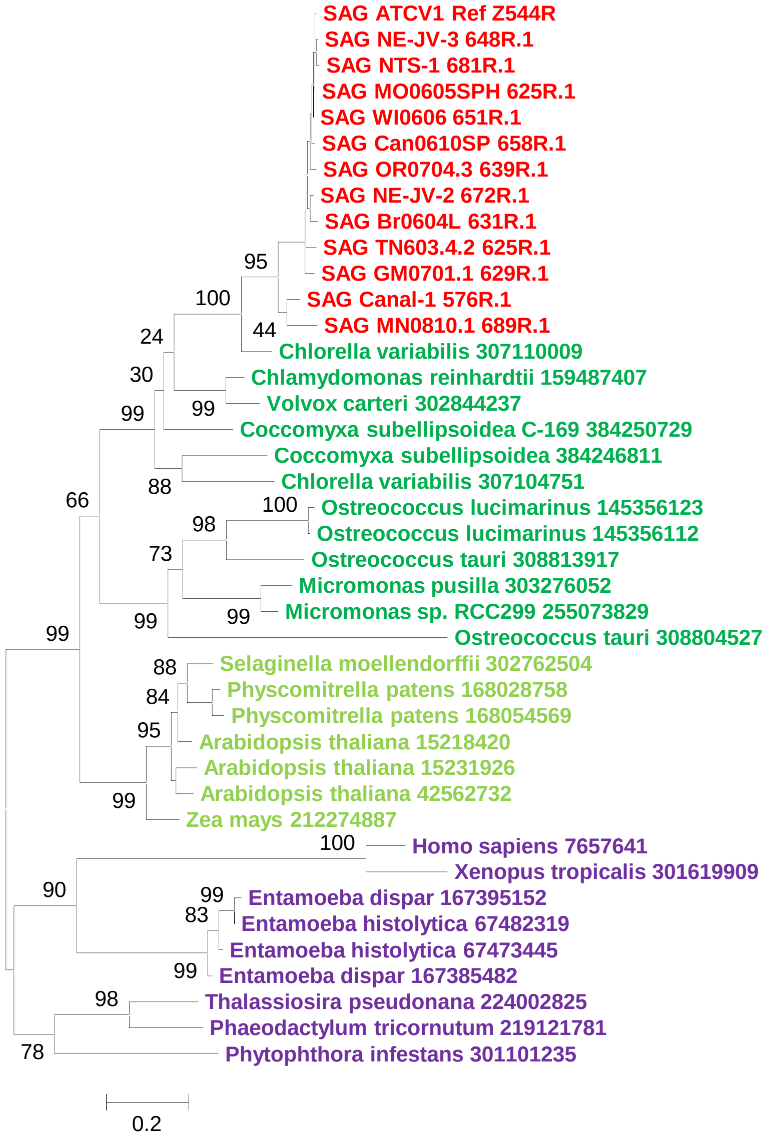
90
82
89
89
0.2

CL0466: MIP family channel protein

CL0780: dUDP-D-glucose 4,6-dehydratase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SAG Canal-1 576R.1
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Volvox carteri 302844237
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Coccomyxa subellipsoidea 384246811
Chlorella variabilis 307104751
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Phaeodactylum tricornutum 219121781
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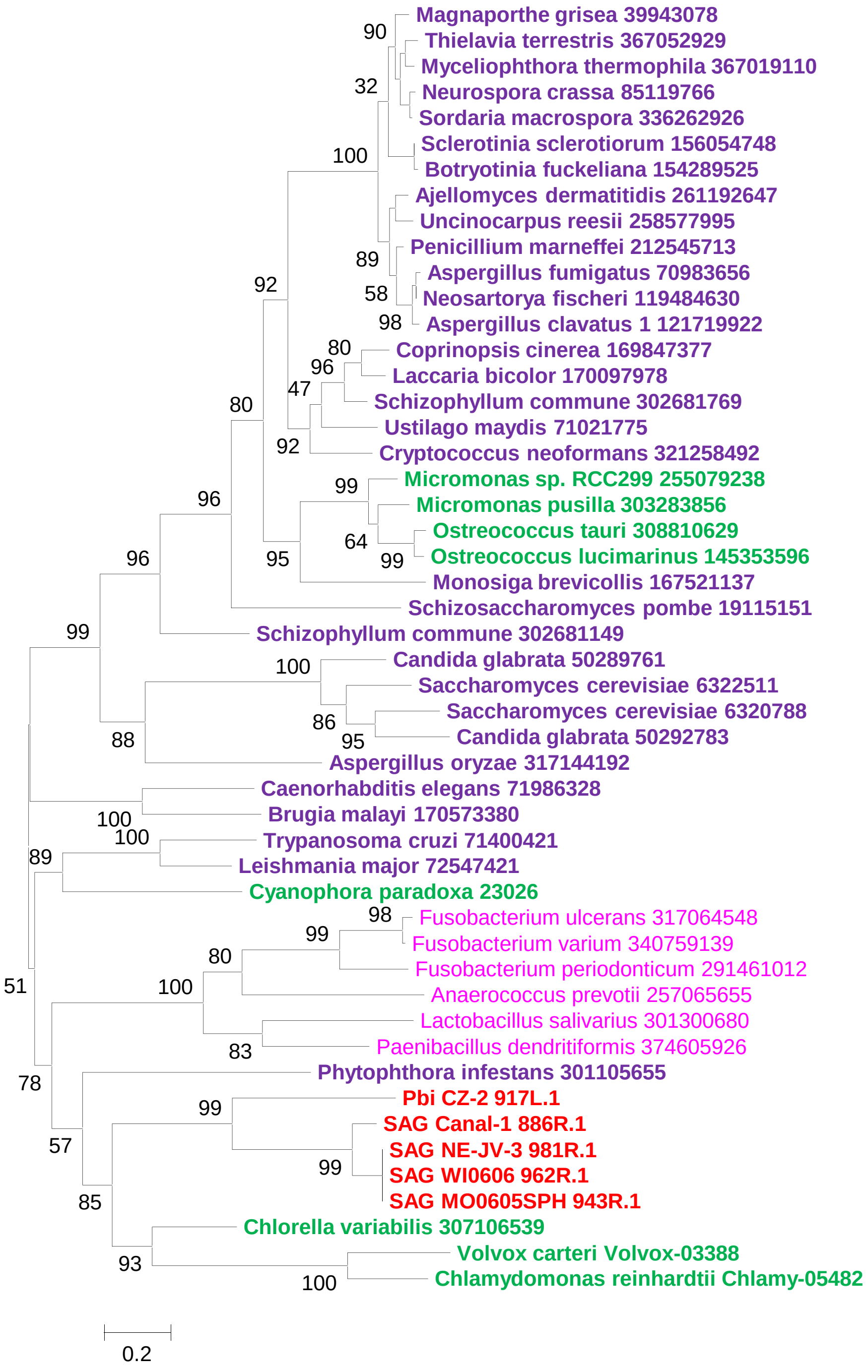
Figure S3: continued

CL0049: NADH-dependent fumarate reductase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Ajellomyces dermatitidis 261192647
Uncinocarpus reesii 258577995
Penicillium marneffei 212545713
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Caenorhabditis elegans 71986328
Brugia malayi 170573380
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Trypanosoma cruzi 71400421
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Leishmania major 72547421
Cyanophora paradoxa 23026
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Fusobacterium ulcerans 317064548
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Fusobacterium varium 340759139
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Fusobacterium periodonticum 291461012
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100
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Lactobacillus salivarius 301300680
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Paenibacillus dendritiformis 374605926
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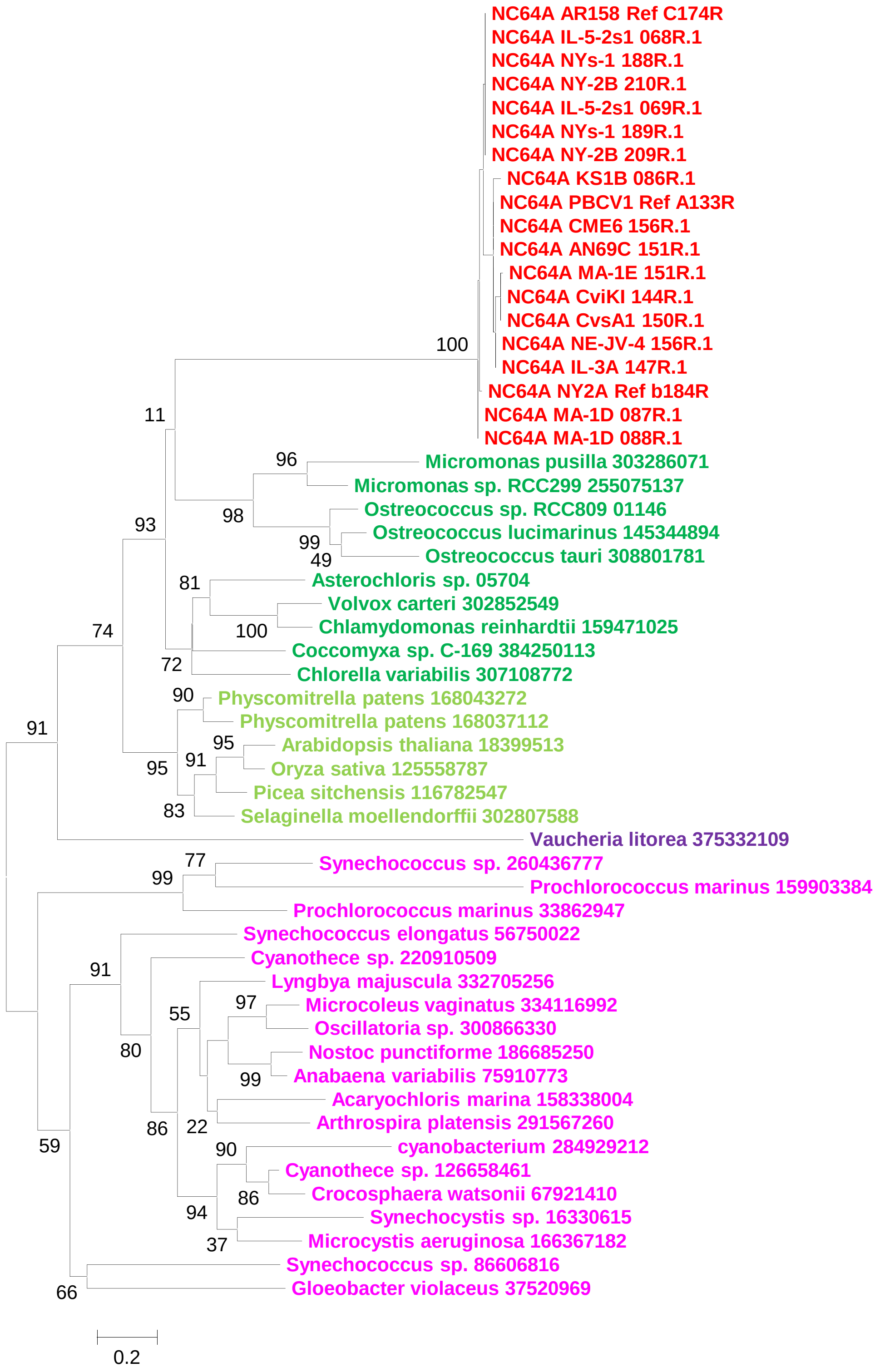
Figure S3: continued

CL0624: THYLAKOID FORMATION 1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Oryza sativa 125558787
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Vaucheria litorea 375332109
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Crocosphaera watsonii 67921410
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Figure S3: continued

CL0778: unknown function

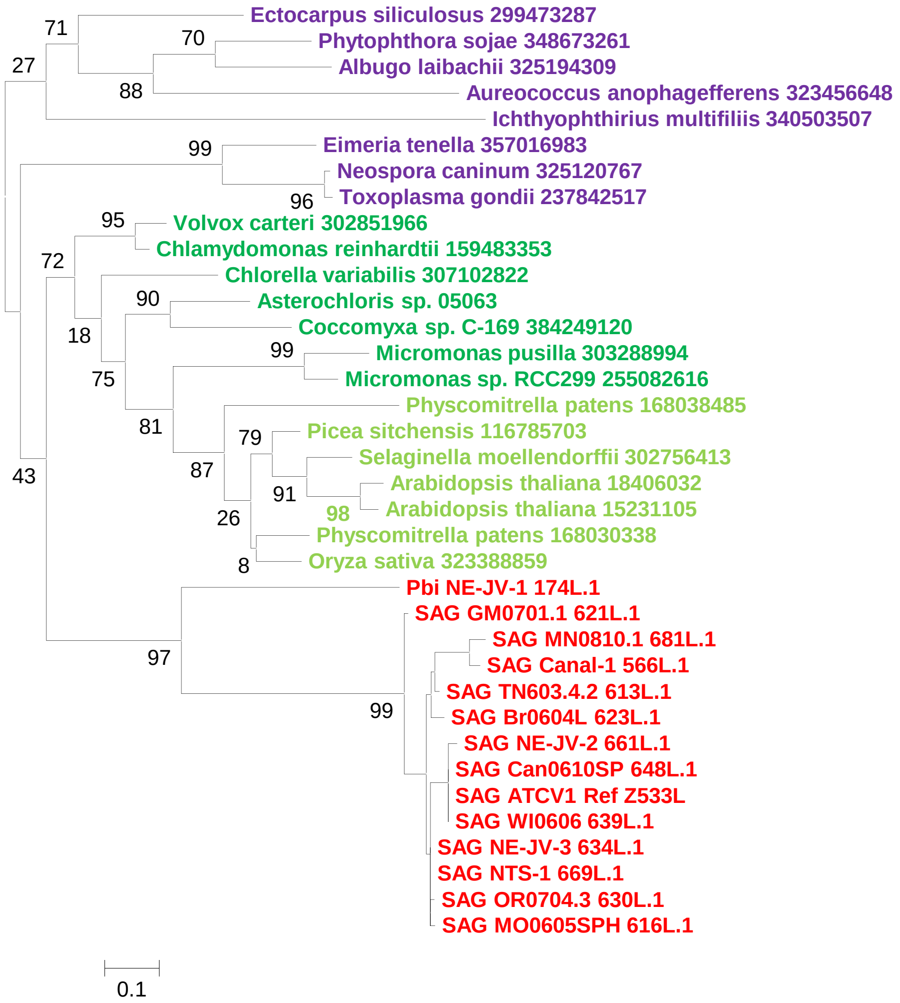

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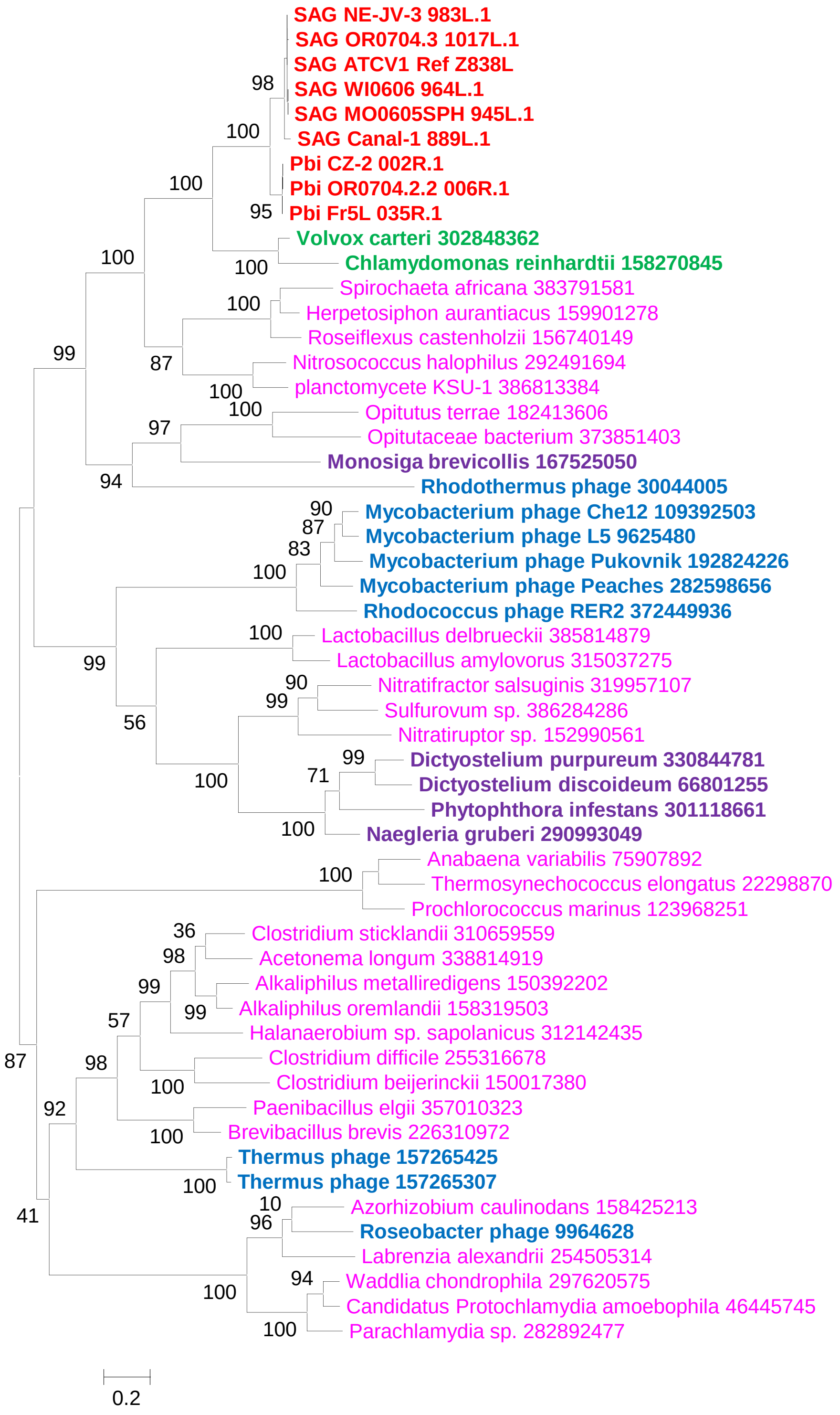
Figure S3: continued

CL0796: ribonucleoside-triphosphate reductase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planctomycete KSU-1 386813384
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Mycobacterium phage Che12 109392503
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Dictyostelium discoideum 66801255
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Thermosynechococcus elongatus 22298870
Prochlorococcus marinus 123968251
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Clostridium beijerinckii 150017380
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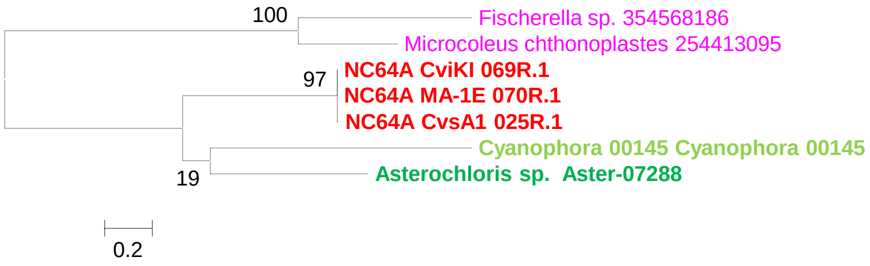
Figure S3: continued

CL0063: unknown function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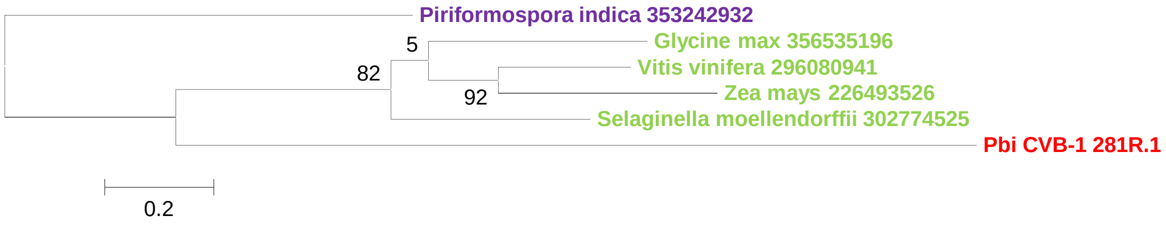
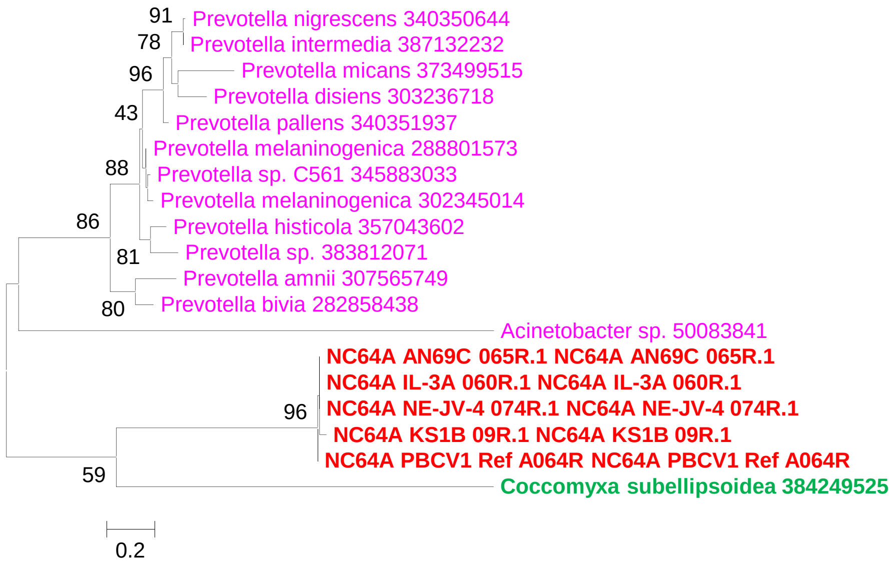
CL0978: unknown function



CL0375: glycosyltransferase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Prevotella sp. C561 345883033
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Prevotella sp. 383812071
Prevotella amnii 307565749
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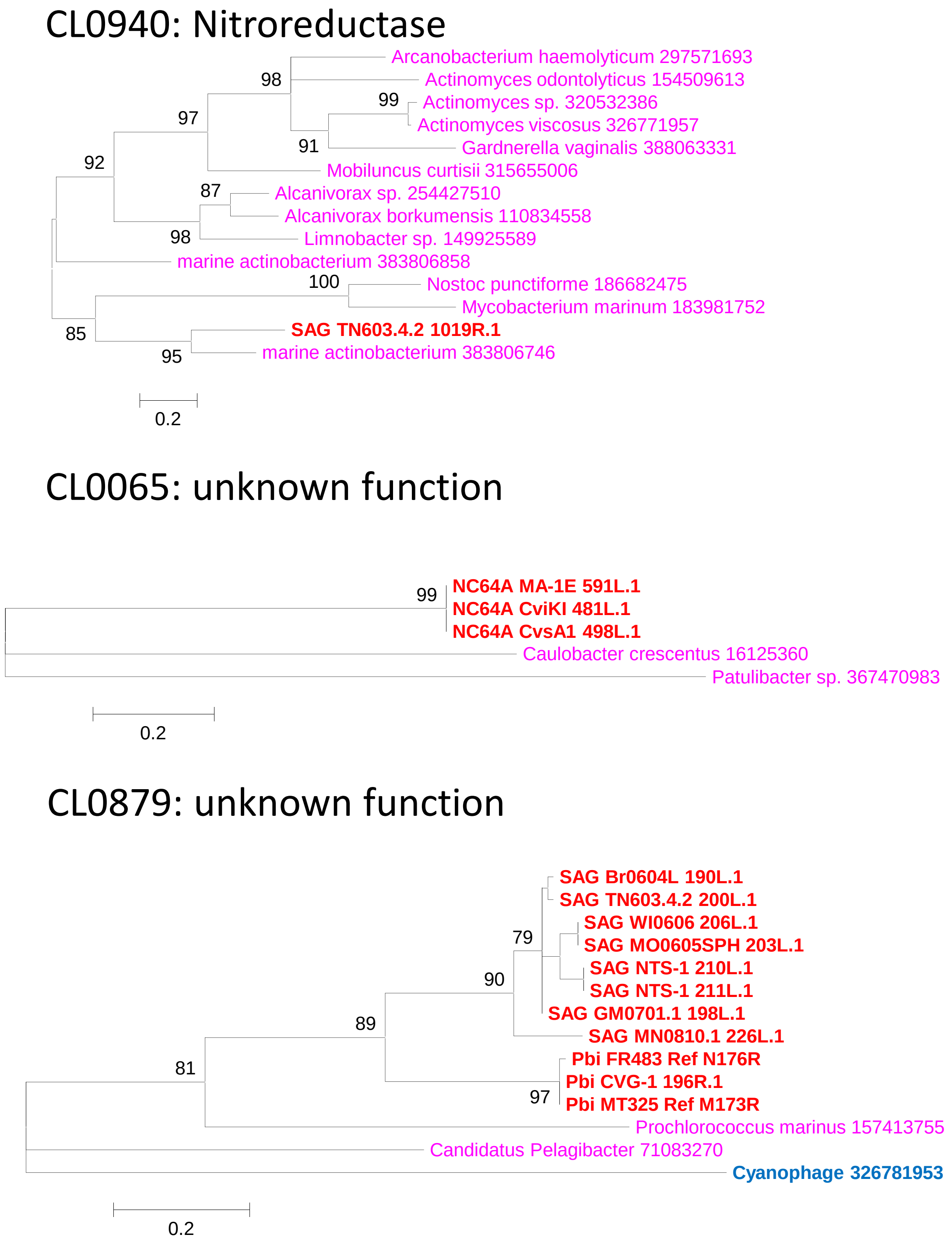
0.2

Figure S3: continued



CL0940: Nitroreductase

Arcanobacterium haemolyticum 297571693
Actinomyces odontolyticus 154509613
Actinomyces sp. 320532386
Actinomyces viscosus 326771957
Gardnerella vaginalis 388063331
Mobiluncus curtisii 315655006
Alcanivorax sp. 254427510
Alcanivorax borkumensis 110834558
Limnobacter sp. 149925589
marine actinobacterium 383806858
Nostoc punctiforme 186682475
Mycobacterium marinum 183981752
SAG TN603.4.2 1019R.1
marine actinobacterium 383806746
98
99
97
91
92
87
98
100
85
95
0.2

CL0065: unknown function

NC64A MA-1E 591L.1
NC64A CviKI 481L.1
NC64A CvsA1 498L.1
Caulobacter crescentus 16125360
Patulibacter sp. 367470983
99
0.2

CL0879: unknown function

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SAG TN603.4.2 200L.1
SAG WI0606 206L.1
SAG MO0605SPH 203L.1
SAG NTS-1 210L.1
SAG NTS-1 211L.1
SAG GM0701.1 198L.1
SAG MN0810.1 226L.1
Pbi FR483 Ref N176R
Pbi CVG-1 196R.1
Pbi MT325 Ref M173R
Prochlorococcus marinus 157413755
Candidatus Pelagibacter 71083270
Cyanophage 326781953
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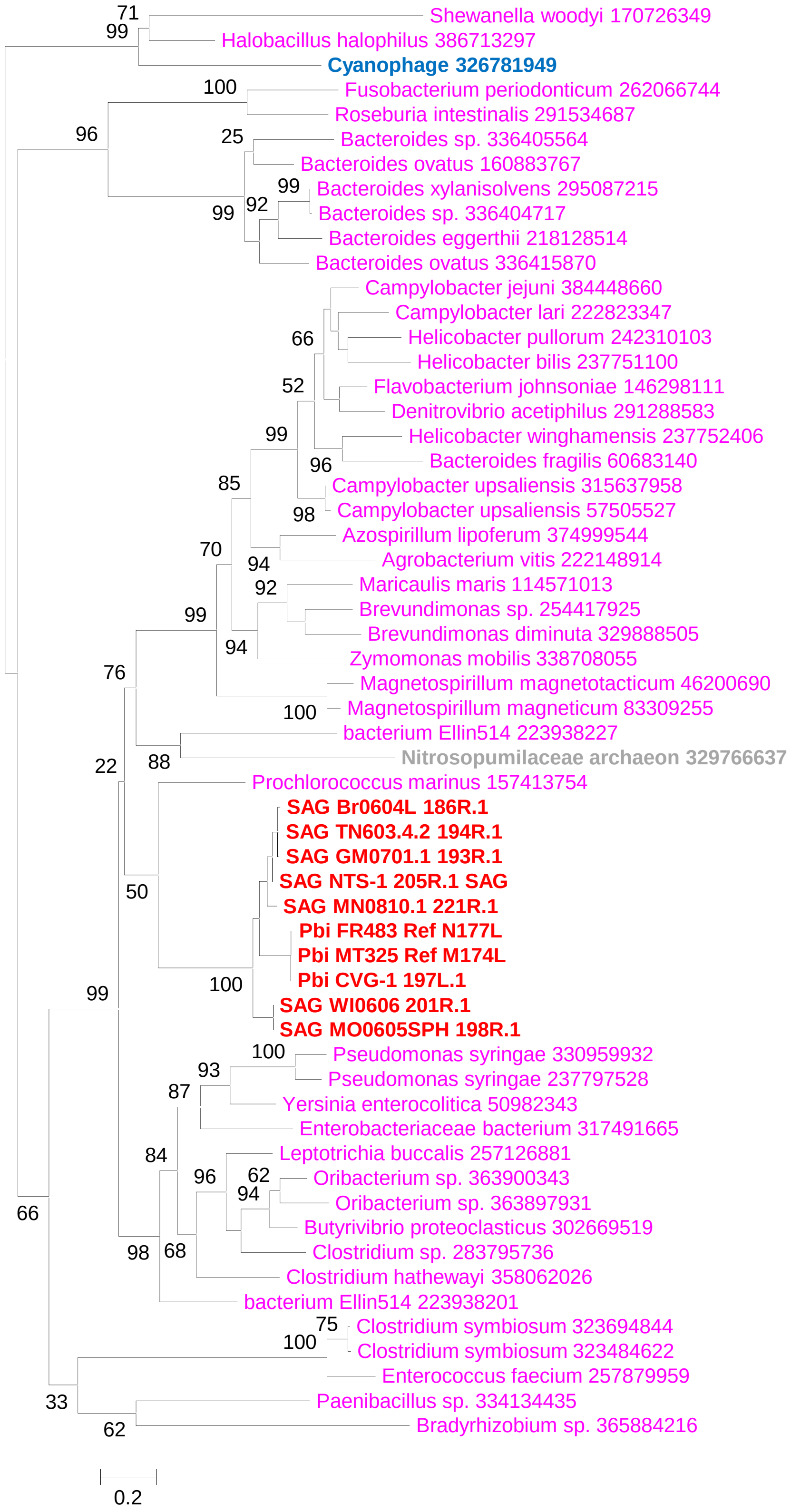
90
89
97
81
0.2

Figure S3: continued

CL0878: dTDP-glucose pyrophosphorylase /HAD superfamily hydrolase


71
99
Shewanella woodyi 170726349
Halobacillus halophilus 386713297
Cyanophage 326781949
100
Fusobacterium periodonticum 262066744
Roseburia intestinalis 291534687
96
25
Bacteroides sp. 336405564
Bacteroides ovatus 160883767
99
Bacteroides xylanisolvens 295087215
99
92
Bacteroides sp. 336404717
Bacteroides eggerthii 218128514
Bacteroides ovatus 336415870
Campylobacter jejuni 384448660
Campylobacter lari 222823347
66
Helicobacter pullorum 242310103
Helicobacter bilis 237751100
52
Flavobacterium johnsoniae 146298111
Denitrovibrio acetiphilus 291288583
99
Helicobacter winghamensis 237752406
96
Bacteroides fragilis 60683140
85
Campylobacter upsaliensis 315637958
98
Campylobacter upsaliensis 57505527
Azospirillum lipoferum 374999544
70
94
Agrobacterium vitis 222148914
92
Maricaulis maris 114571013
99
Brevundimonas sp. 254417925
Brevundimonas diminuta 329888505
94
Zymomonas mobilis 338708055
76
Magnetospirillum magnetotacticum 46200690
100
Magnetospirillum magneticum 83309255
bacterium Ellin514 223938227
22
88
Nitrosopumilaceae archaeon 329766637
Prochlorococcus marinus 157413754
SAG Br0604L 186R.1
SAG TN603.4.2 194R.1
SAG GM0701.1 193R.1
SAG NTS-1 205R.1 SAG
50
SAG MN0810.1 221R.1
Pbi FR483 Ref N177L
Pbi MT325 Ref M174L
100
Pbi CVG-1 197L.1
99
SAG WI0606 201R.1
SAG MO0605SPH 198R.1
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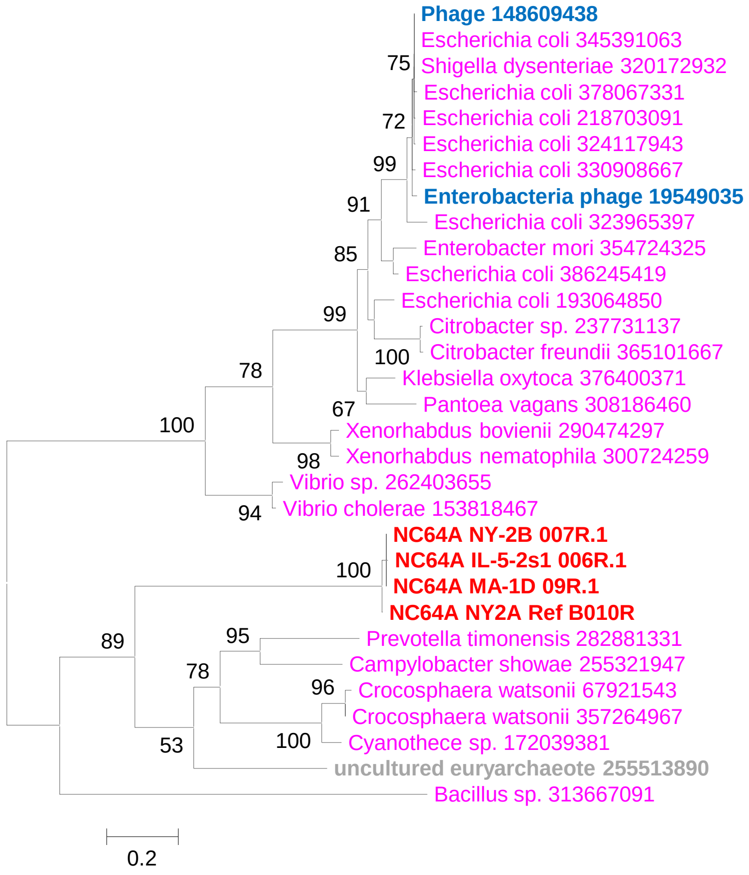
93
Pseudomonas syringae 237797528
87
Yersinia enterocolitica 50982343
Enterobacteriaceae bacterium 317491665
84
Leptotrichia buccalis 257126881
96
62
Oribacterium sp. 363900343
94
Oribacterium sp. 363897931
66
Butyrivibrio proteoclasticus 302669519
98
68
Clostridium sp. 283795736
Clostridium hathewayi 358062026
bacterium Ellin514 223938201
75
Clostridium symbiosum 323694844
100
Clostridium symbiosum 323484622
Enterococcus faecium 257879959
33
Paenibacillus sp. 334134435
62
Bradyrhizobium sp. 365884216
0.2

Figure S3: continued

CL0222: DNA methylase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Escherichia coli 345391063
Shigella dysenteriae 320172932
Escherichia coli 378067331
Escherichia coli 218703091
Escherichia coli 324117943
Escherichia coli 330908667
Enterobacteria phage 19549035
Escherichia coli 323965397
Enterobacter mori 354724325
Escherichia coli 386245419
Escherichia coli 193064850
Citrobacter sp. 237731137
Citrobacter freundii 365101667
Klebsiella oxytoca 376400371
Pantoea vagans 308186460
Xenorhabdus bovienii 290474297
Xenorhabdus nematophila 300724259
Vibrio sp. 262403655
Vibrio cholerae 153818467
NC64A NY-2B 007R.1
NC64A IL-5-2s1 006R.1
NC64A MA-1D 09R.1
NC64A NY2A Ref B010R
Prevotella timonensis 282881331
Campylobacter showae 255321947
Crocosphaera watsonii 67921543
Crocosphaera watsonii 357264967
Cyanothece sp. 172039381
uncultured euryarchaeote 255513890
Bacillus sp. 313667091
75
72
99
91
85
99
100
78
67
100
98
94
100
89
95
78
96
53
100
0.2

CL0792: mannose-6-phosphate isomerase

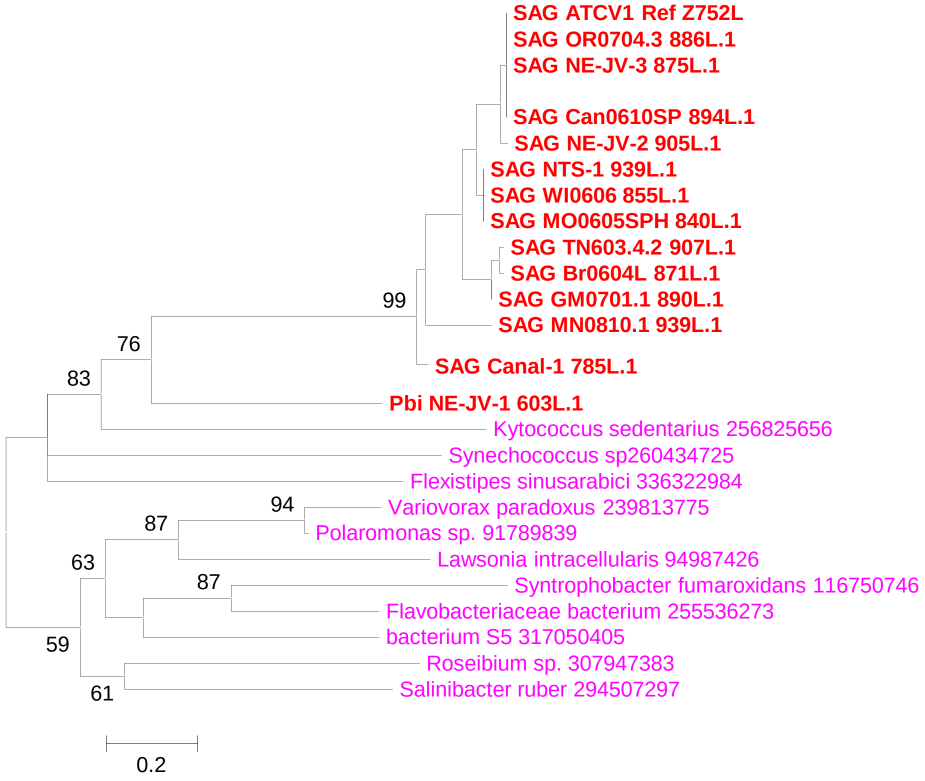
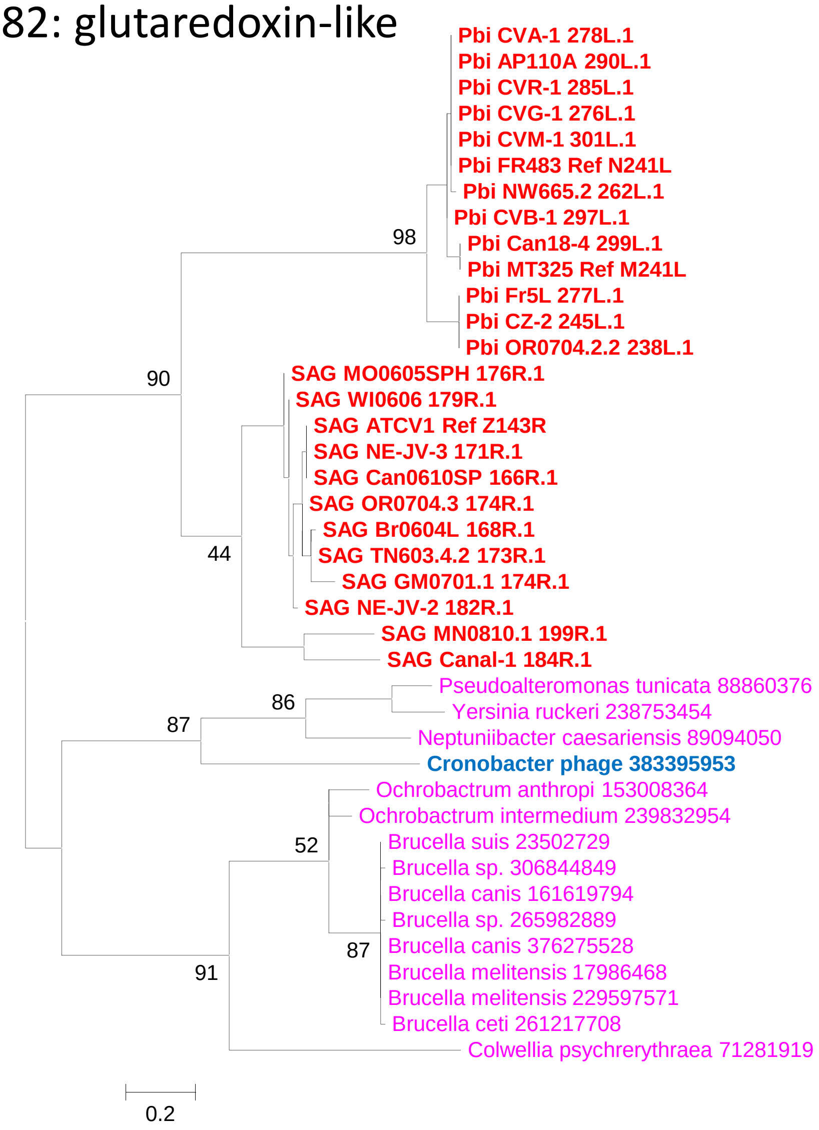


Figure S3: continued

CL0482: glutaredoxin-like



CL0055: unknown function

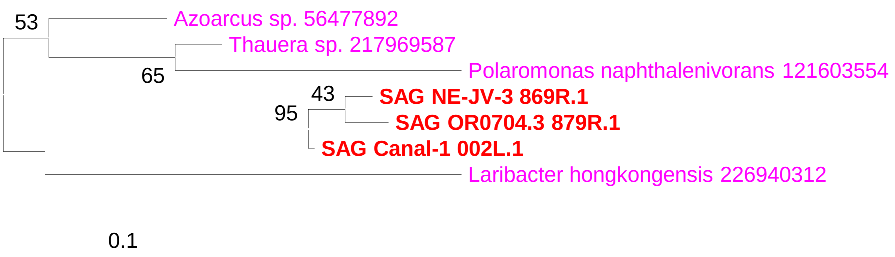
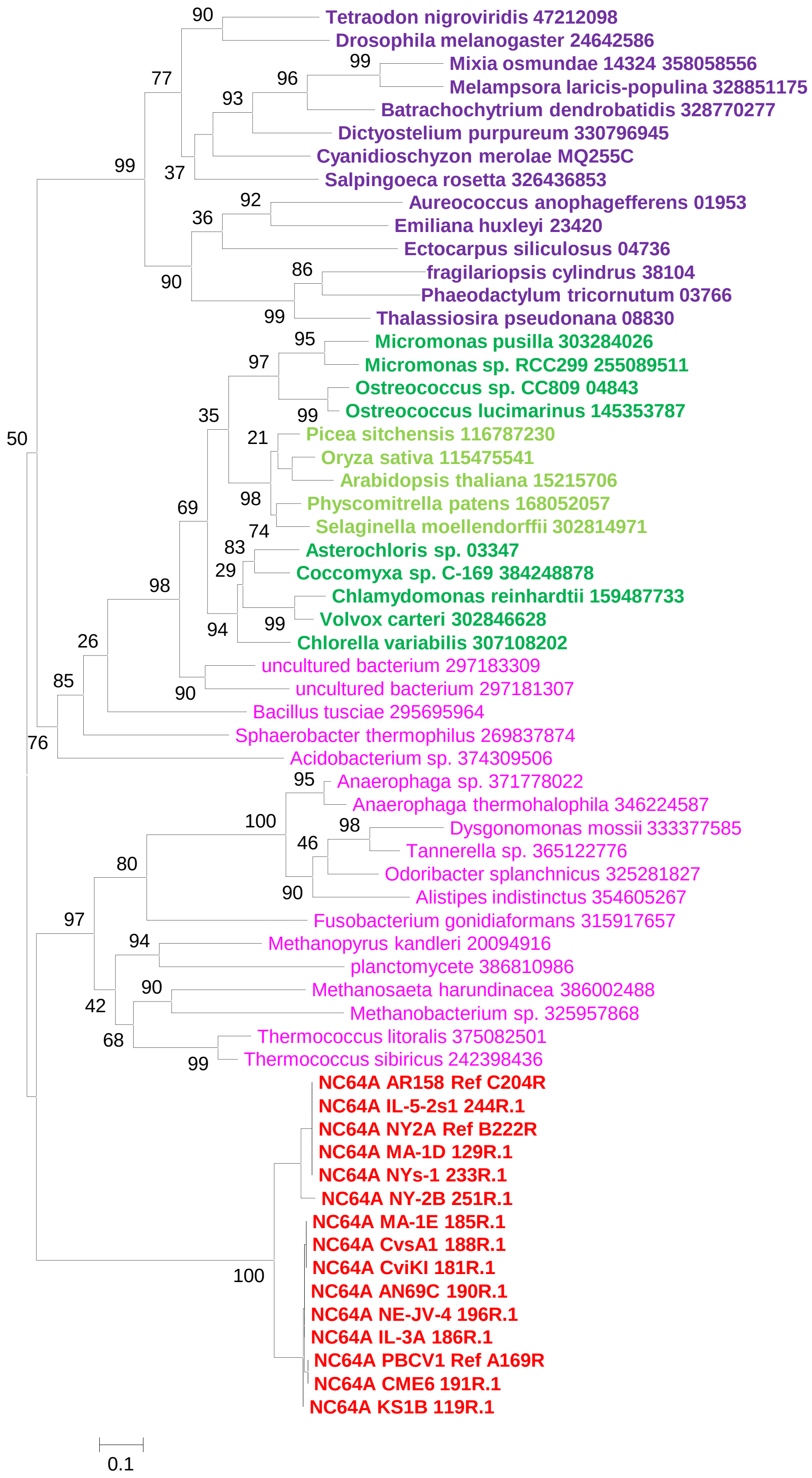


Figure S3: continued

CL0739: aspartate carbamoyltransferase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Drosophila melanogaster 24642586
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Mixia osmundae 14324 358058556
77
96
Melampsora laricis-populina 328851175
93
Batrachochytrium dendrobatidis 328770277
Dictyostelium purpureum 330796945
Cyanidioschyzon merolae MQ255C
99
37
Salpingoeca rosetta 326436853
92
Aureococcus anophagefferens 01953
36
Emiliana huxleyi 23420
Ectocarpus siliculosus 04736
86
fragilariopsis cylindrus 38104
90
Phaeodactylum tricornutum 03766
99
Thalassiosira pseudonana 08830
95
Micromonas pusilla 303284026
97
Micromonas sp. RCC299 255089511
Ostreococcus sp. CC809 04843
35
99
Ostreococcus lucimarinus 145353787
50
21
Picea sitchensis 116787230
Oryza sativa 115475541
Arabidopsis thaliana 15215706
98
Physcomitrella patens 168052057
69
Selaginella moellendorffii 302814971
74
83
Asterochloris sp. 03347
29
Coccomyxa sp. C-169 384248878
98
Chlamydomonas reinhardtii 159487733
99
94
Volvox carteri 302846628
26
Chlorella variabilis 307108202
uncultured bacterium 297183309
85
90
uncultured bacterium 297181307
Bacillus tusciae 295695964
76
Sphaerobacter thermophilus 269837874
Acidobacterium sp. 374309506
95
Anaerophaga sp. 371778022
Anaerophaga thermohalophila 346224587
100
98
Dysgonomonas mossii 333377585
46
Tannerella sp. 365122776
80
Odoribacter splanchnicus 325281827
90
Alistipes indistinctus 354605267
97
Fusobacterium gonidiaformans 315917657
94
Methanopyrus kandleri 20094916
planctomycete 386810986
90
Methanosaeta harundinacea 386002488
42
Methanobacterium sp. 325957868
68
Thermococcus litoralis 375082501
99
Thermococcus sibiricus 242398436
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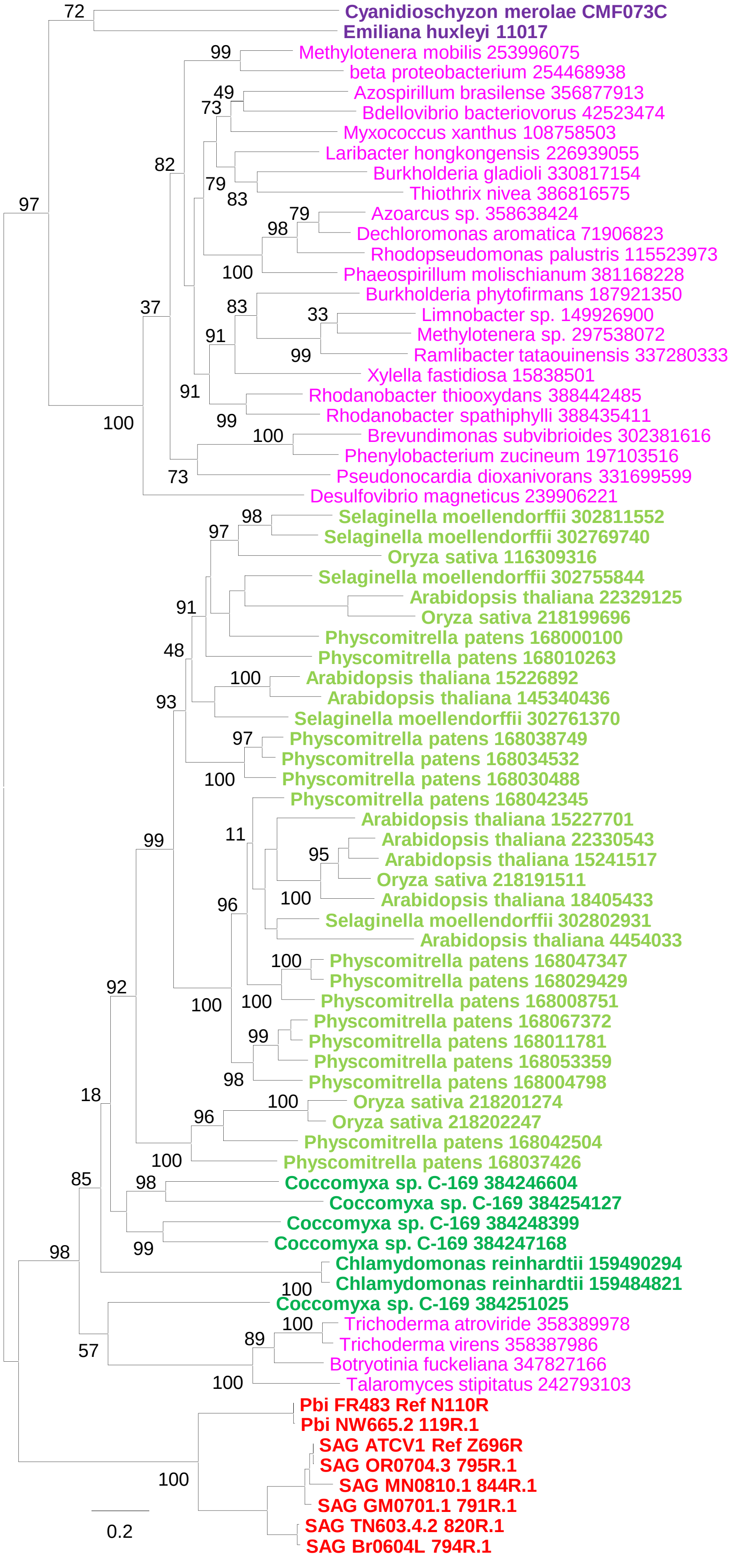
NC64A NY2A Ref B222R
NC64A MA-1D 129R.1
NC64A NYs-1 233R.1
NC64A NY-2B 251R.1
NC64A MA-1E 185R.1
NC64A CvsA1 188R.1
NC64A CviKI 181R.1
100
NC64A AN69C 190R.1
NC64A NE-JV-4 196R.1
NC64A IL-3A 186R.1
NC64A PBCV1 Ref A169R
NC64A CME6 191R.1
NC64A KS1B 119R.1
0.1

Figure S3: continued

CL0787:Potassium transporter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Cyanidioschyzon merolae CMF073C
Emiliana huxleyi 11017
99
Methylotenera mobilis 253996075
beta proteobacterium 254468938
49
Azospirillum brasilense 356877913
73
Bdellovibrio bacteriovorus 42523474
Myxococcus xanthus 108758503
82
Laribacter hongkongensis 226939055
Burkholderia gladioli 330817154
79
97
Thiothrix nivea 386816575
83
79
Azoarcus sp. 358638424
98
Dechloromonas aromatica 71906823
Rhodopseudomonas palustris 115523973
100
Phaeospirillum molischianum 381168228
Burkholderia phytofirmans 187921350
37
83
33
Limnobacter sp. 149926900
91
Methylotenera sp. 297538072
99
Ramlibacter tataouinensis 337280333
Xylella fastidiosa 15838501
91
Rhodanobacter thiooxydans 388442485
100
99
Rhodanobacter spathiphylli 388435411
100
Brevundimonas subvibrioides 302381616
Phenylobacterium zucineum 197103516
73
Pseudonocardia dioxanivorans 331699599
Desulfovibrio magneticus 239906221
98
Selaginella moellendorffii 302811552
97
Selaginella moellendorffii 302769740
Oryza sativa 116309316
Selaginella moellendorffii 302755844
Arabidopsis thaliana 22329125
91
Oryza sativa 218199696
Physcomitrella patens 168000100
48
Physcomitrella patens 168010263
100
Arabidopsis thaliana 15226892
93
Arabidopsis thaliana 145340436
Selaginella moellendorffii 302761370
97
Physcomitrella patens 168038749
Physcomitrella patens 168034532
100
Physcomitrella patens 168030488
Physcomitrella patens 168042345
11
Arabidopsis thaliana 15227701
99
Arabidopsis thaliana 22330543
95
Arabidopsis thaliana 15241517
Oryza sativa 218191511
100
Arabidopsis thaliana 18405433
96
Selaginella moellendorffii 302802931
Arabidopsis thaliana 4454033
100
Physcomitrella patens 168047347
Physcomitrella patens 168029429
92
100
100
Physcomitrella patens 168008751
Physcomitrella patens 168067372
99
Physcomitrella patens 168011781
Physcomitrella patens 168053359
98
Physcomitrella patens 168004798
18
100
Oryza sativa 218201274
96
Oryza sativa 218202247
Physcomitrella patens 168042504
100
Physcomitrella patens 168037426
85
98
Coccomyxa sp. C-169 384246604
Coccomyxa sp. C-169 384254127
Coccomyxa sp. C-169 384248399
99
Coccomyxa sp. C-169 384247168
98
Chlamydomonas reinhardtii 159490294
100
Chlamydomonas reinhardtii 159484821
Coccomyxa sp. C-169 384251025
100
Trichoderma atroviride 358389978
89
57
Trichoderma virens 358387986
Botryotinia fuckeliana 347827166
100
Talaromyces stipitatus 242793103
Pbi FR483 Ref N110R
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SAG ATCV1 Ref Z696R
SAG OR0704.3 795R.1
SAG MN0810.1 844R.1
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SAG TN603.4.2 820R.1
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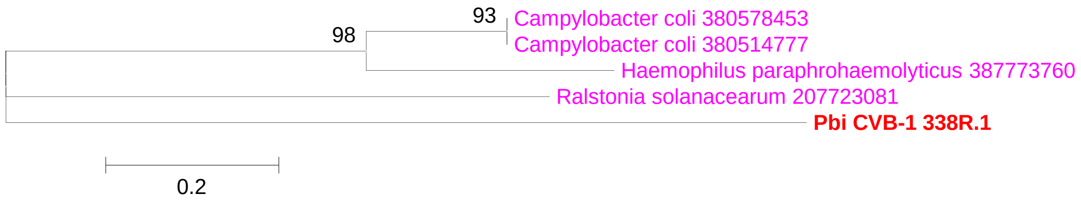
SAG Br0604L 794R.1

Figure S3: continued

CL0989: unknown function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98
Campylobacter coli 380578453
Campylobacter coli 380514777
Haemophilus paraphrohaemolyticus 387773760
Ralstonia solanacearum 207723081
Pbi CVB-1 338R.1
0.2

CL0767: unknown function

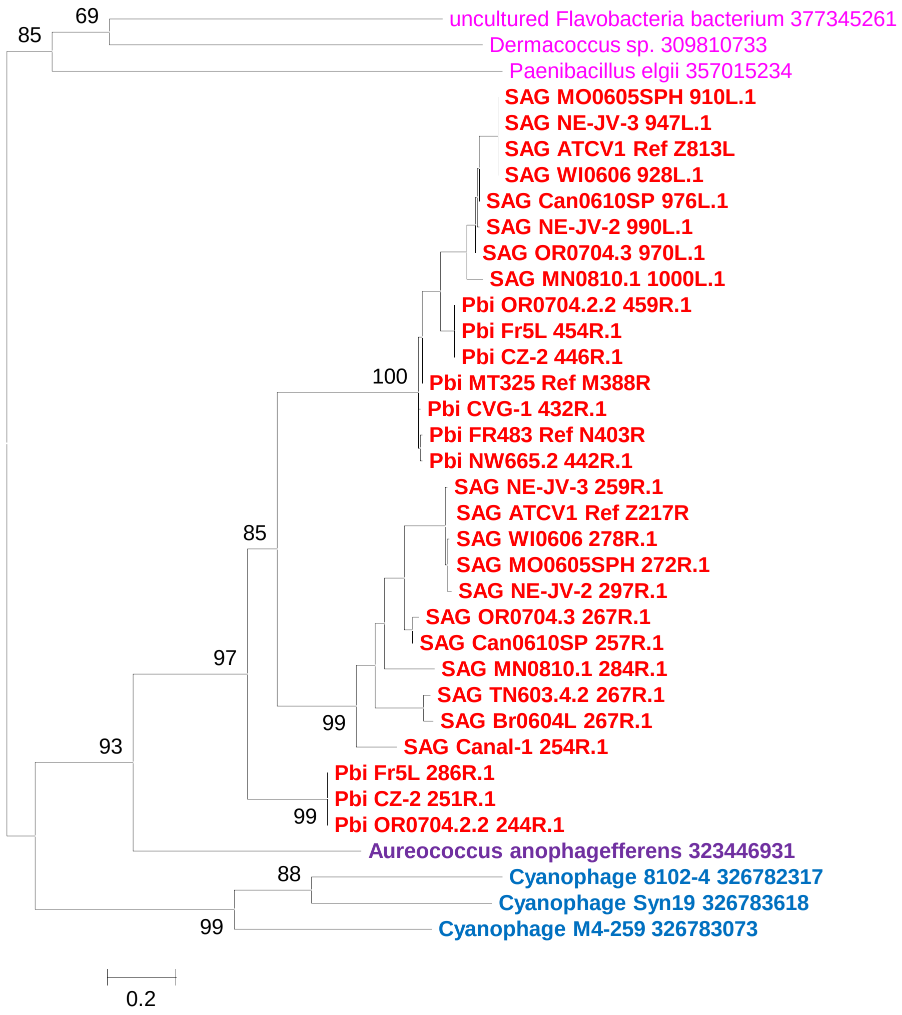
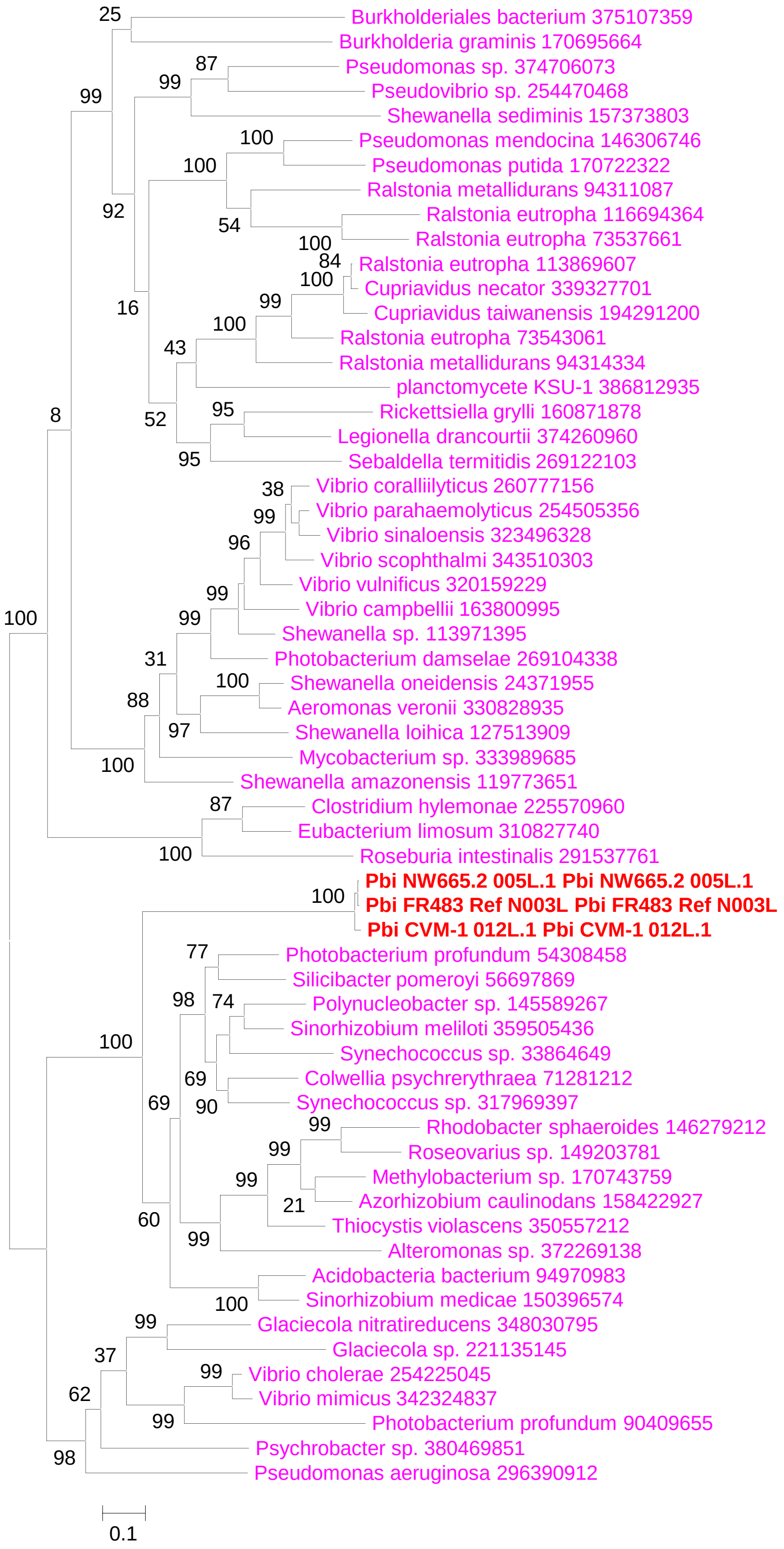


Figure S3: continued

CL0037: beta-lactamase-like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Burkholderia graminis 170695664
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Pseudomonas sp. 374706073
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Pseudovibrio sp. 254470468
99
Shewanella sediminis 157373803
100
Pseudomonas mendocina 146306746
100
Pseudomonas putida 170722322
Ralstonia metallidurans 94311087
92
54
Ralstonia eutropha 116694364
Ralstonia eutropha 73537661
100
84
Ralstonia eutropha 113869607
100
Cupriavidus necator 339327701
16
Cupriavidus taiwanensis 194291200
99
100
Ralstonia eutropha 73543061
43
Ralstonia metallidurans 94314334
planctomycete KSU-1 386812935
8
95
Rickettsiella grylli 160871878
52
Legionella drancourtii 374260960
95
Sebaldella termitidis 269122103
38
Vibrio coralliilyticus 260777156
99
Vibrio parahaemolyticus 254505356
96
Vibrio sinaloensis 323496328
Vibrio scophthalmi 343510303
Vibrio vulnificus 320159229
99
Vibrio campbellii 163800995
100
99
Shewanella sp. 113971395
Photobacterium damselae 269104338
31
100
Shewanella oneidensis 24371955
88
Aeromonas veronii 330828935
97
Shewanella loihica 127513909
Mycobacterium sp. 333989685
100
Shewanella amazonensis 119773651
87
Clostridium hylemonae 225570960
Eubacterium limosum 310827740
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Roseburia intestinalis 291537761
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100
Pbi FR483 Ref N003L Pbi FR483 Ref N003L
Pbi CVM-1 012L.1 Pbi CVM-1 012L.1
77
Photobacterium profundum 54308458
Silicibacter pomeroyi 56697869
98
74
Polynucleobacter sp. 145589267
Sinorhizobium meliloti 359505436
100
Synechococcus sp. 33864649
69
Colwellia psychrerythraea 71281212
69
90
Synechococcus sp. 317969397
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Rhodobacter sphaeroides 146279212
99
Roseovarius sp. 149203781
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Methylobacterium sp. 170743759
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Azorhizobium caulinodans 158422927
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Thiocystis violascens 350557212
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Alteromonas sp. 372269138
Acidobacteria bacterium 94970983
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Sinorhizobium medicae 150396574
99
Glaciecola nitratireducens 348030795
Glaciecola sp. 221135145
37
99
Vibrio cholerae 254225045
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Vibrio mimicus 342324837
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Photobacterium profundum 90409655
Psychrobacter sp. 380469851
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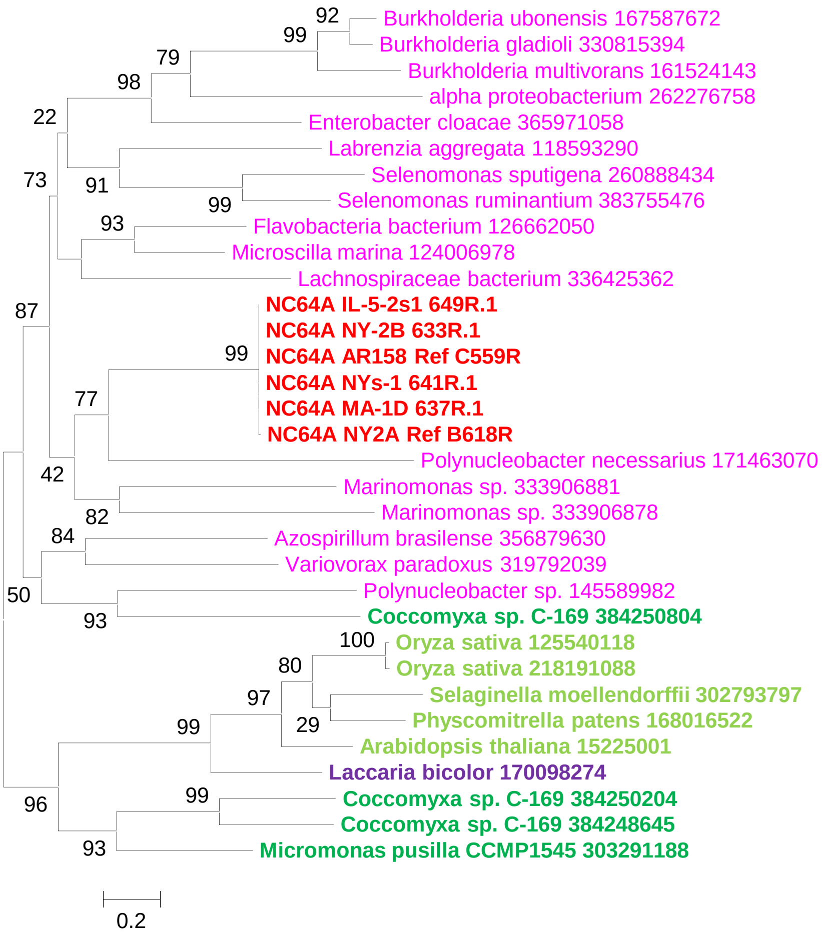
Pseudomonas aeruginosa 296390912
0.1

Figure S3: continued

CL0561: Glycosyltransferase family 17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Burkholderia gladioli 330815394
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Burkholderia multivorans 161524143
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alpha proteobacterium 262276758
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Enterobacter cloacae 365971058
Labrenzia aggregata 118593290
73
91
Selenomonas sputigena 260888434
99
Selenomonas ruminantium 383755476
93
Flavobacteria bacterium 126662050
Microscilla marina 124006978
Lachnospiraceae bacterium 336425362
87
NC64A IL-5-2s1 649R.1
NC64A NY-2B 633R.1
99
NC64A AR158 Ref C559R
NC64A NYs-1 641R.1
77
NC64A MA-1D 637R.1
NC64A NY2A Ref B618R
Polynucleobacter necessarius 171463070
42
Marinomonas sp. 333906881
82
Marinomonas sp. 333906878
84
Azospirillum brasilense 356879630
Variovorax paradoxus 319792039
50
Polynucleobacter sp. 145589982
93
Coccomyxa sp. C-169 384250804
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Oryza sativa 125540118
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Oryza sativa 218191088
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Selaginella moellendorffii 302793797
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Physcomitrella patens 168016522
Arabidopsis thaliana 15225001
Laccaria bicolor 170098274
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96
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Coccomyxa sp. C-169 384248645
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Micromonas pusilla CCMP1545 303291188
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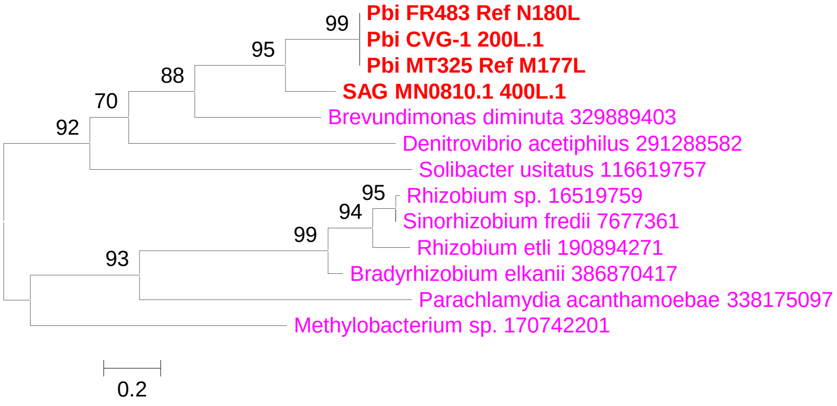
CL0876: methyltransferase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Denitrovibrio acetiphilus 291288582
Solibacter usitatus 116619757
95
Rhizobium sp. 16519759
94
Sinorhizobium fredii 7677361
99
Rhizobium etli 190894271
93
Bradyrhizobium elkanii 386870417
Parachlamydia acanthamoebae 338175097
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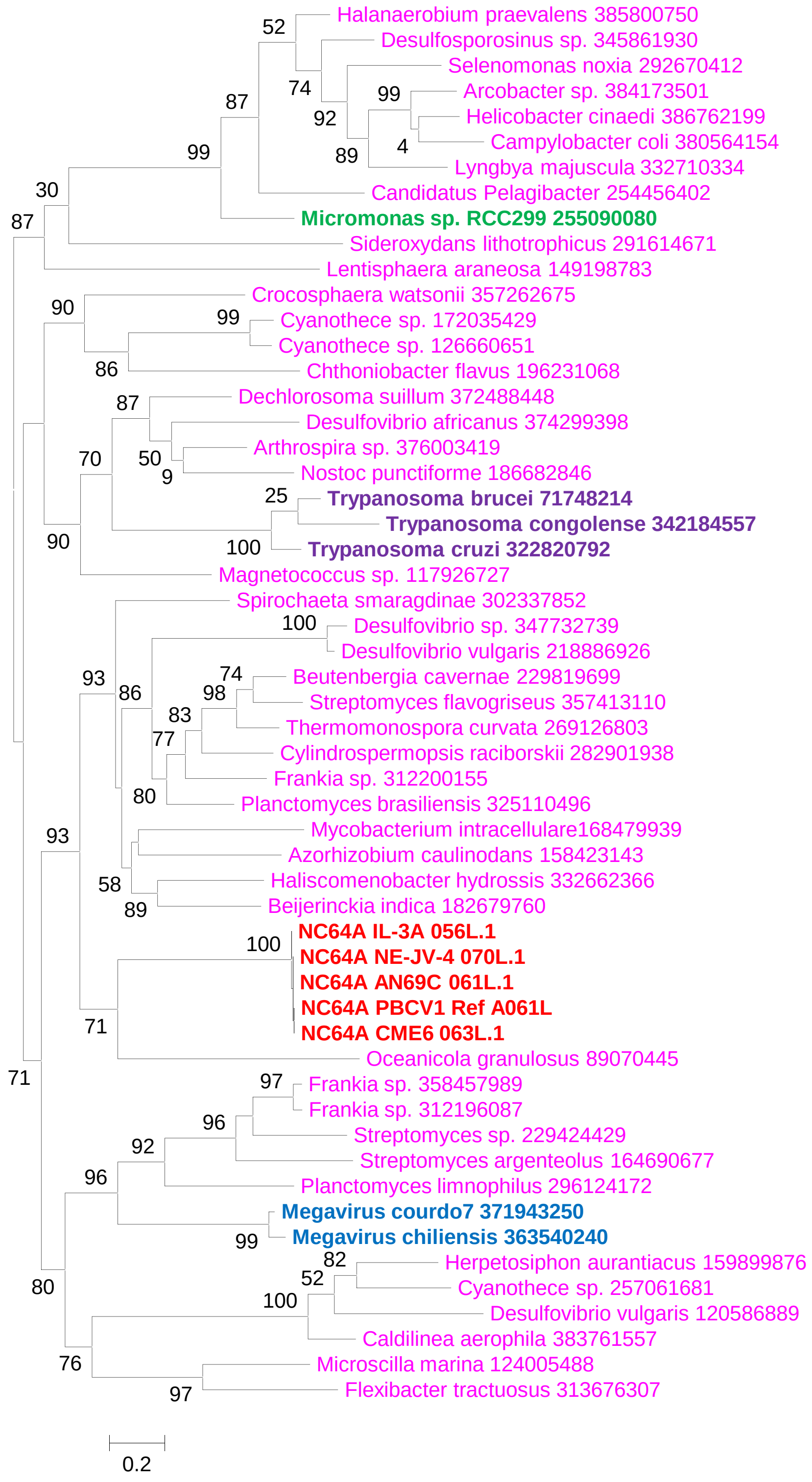
0.2

Figure S3: continued

CL0356: methyltransferase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Campylobacter coli 380564154
Lyngbya majuscula 332710334
Candidatus Pelagibacter 254456402
Micromonas sp. RCC299 255090080
Sideroxydans lithotrophicus 291614671
Lentisphaera araneosa 149198783
Crocosphaera watsonii 357262675
Cyanothece sp. 172035429
Cyanothece sp. 126660651
Chthoniobacter flavus 196231068
Dechlorosoma suillum 372488448
Desulfovibrio africanus 374299398
Arthrospira sp. 376003419
Nostoc punctiforme 186682846
Trypanosoma brucei 71748214
Trypanosoma congolense 342184557
Trypanosoma cruzi 322820792
Magnetococcus sp. 117926727
Spirochaeta smaragdinae 302337852
Desulfovibrio sp. 347732739
Desulfovibrio vulgaris 218886926
Beutenbergia cavernae 229819699
Streptomyces flavogriseus 357413110
Thermomonospora curvata 269126803
Cylindrospermopsis raciborskii 282901938
Frankia sp. 312200155
Planctomyces brasiliensis 325110496
Mycobacterium intracellulare168479939
Azorhizobium caulinodans 158423143
Haliscomenobacter hydrossis 332662366
Beijerinckia indica 182679760
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NC64A NE-JV-4 070L.1
NC64A AN69C 061L.1
NC64A PBCV1 Ref A061L
NC64A CME6 063L.1
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Frankia sp. 358457989
Frankia sp. 312196087
Streptomyces sp. 229424429
Streptomyces argenteolus 164690677
Planctomyces limnophilus 296124172
Megavirus courdo7 371943250
Megavirus chiliensis 363540240
Herpetosiphon aurantiacus 159899876
Cyanothece sp. 257061681
Desulfovibrio vulgaris 120586889
Caldilinea aerophila 383761557
Microscilla marina 124005488
Flexibacter tractuosus 313676307
52
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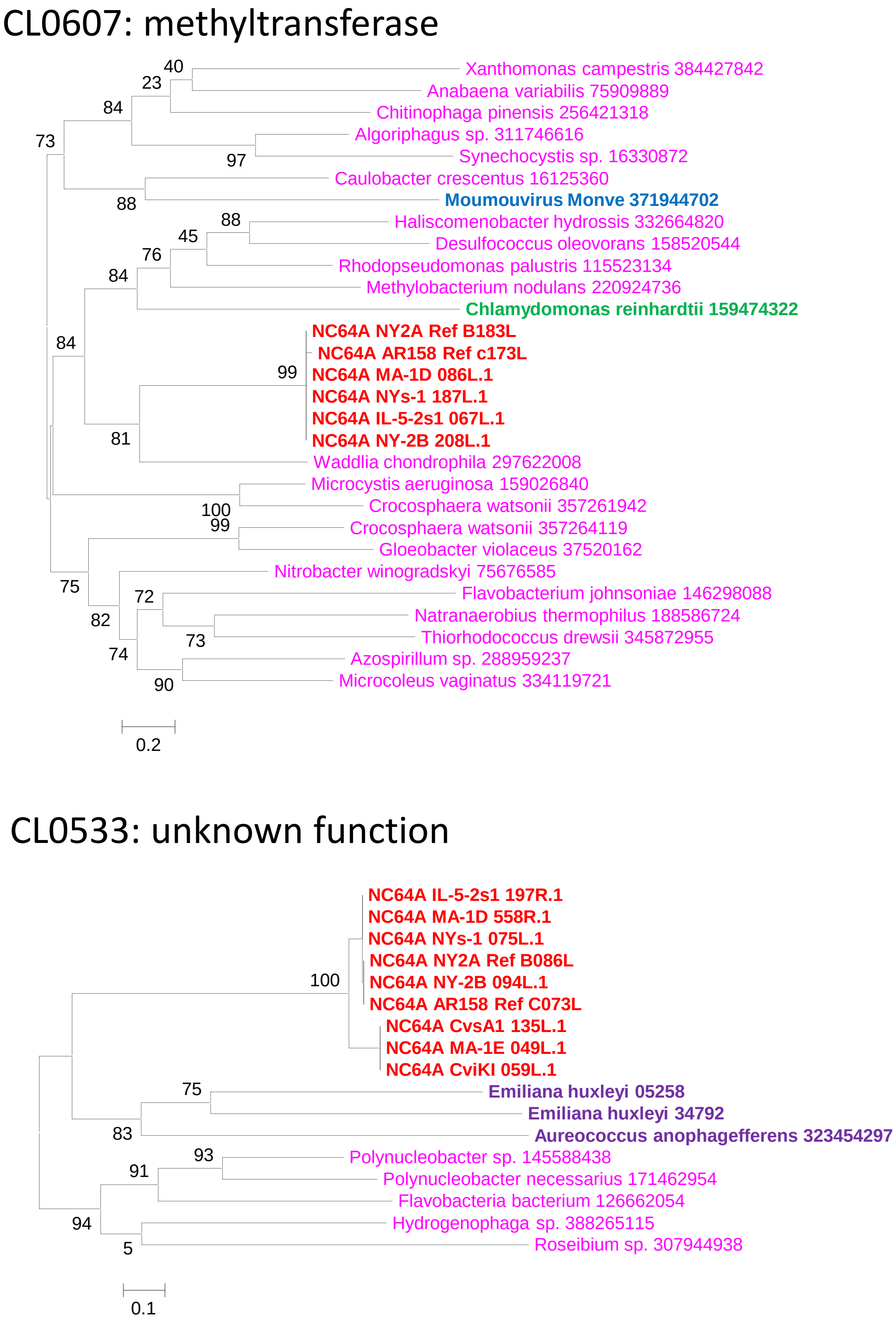
0.2

Figure S3: continued



CL0607: methyltransferase

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97
88
88
45
76
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84
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82
73
74
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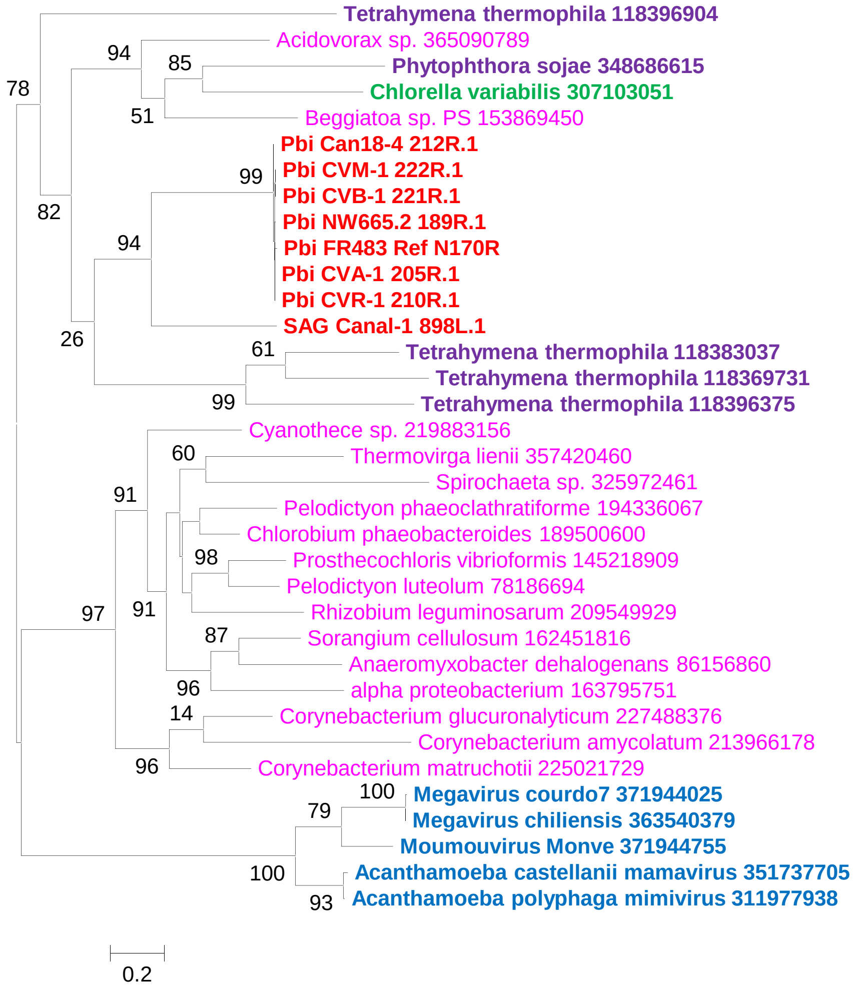
Xanthomonas campestris 384427842
Anabaena variabilis 75909889
Chitinophaga pinensis 256421318
Algoriphagus sp. 311746616
Synechocystis sp. 16330872
Caulobacter crescentus 16125360
Moumouvirus Monve 371944702
Haliscomenobacter hydrossis 332664820
Desulfococcus oleovorans 158520544
Rhodopseudomonas palustris 115523134
Methylobacterium nodulans 220924736
Chlamydomonas reinhardtii 159474322
NC64A NY2A Ref B183L
NC64A AR158 Ref c173L
NC64A MA-1D 086L.1
NC64A NYs-1 187L.1
NC64A IL-5-2s1 067L.1
NC64A NY-2B 208L.1
Waddlia chondrophila 297622008
Microcystis aeruginosa 159026840
Crocosphaera watsonii 357261942
Crocosphaera watsonii 357264119
Gloeobacter violaceus 37520162
Nitrobacter winogradskyi 75676585
Flavobacterium johnsoniae 146298088
Natranaerobius thermophilus 188586724
Thiorhodococcus drewsii 345872955
Azospirillum sp. 288959237
Microcoleus vaginatus 334119721
0.2

CL0533: unknown function

NC64A IL-5-2s1 197R.1
NC64A MA-1D 558R.1
NC64A NYs-1 075L.1
NC64A NY2A Ref B086L
NC64A NY-2B 094L.1
NC64A AR158 Ref C073L
NC64A CvsA1 135L.1
NC64A MA-1E 049L.1
NC64A CviKI 059L.1
100
75
Emiliana huxleyi 05258
Emiliana huxleyi 34792
Aureococcus anophagefferens 323454297
83
93
91
Polynucleobacter sp. 145588438
Polynucleobacter necessarius 171462954
Flavobacteria bacterium 126662054
94
Hydrogenophaga sp. 388265115
5
Roseibium sp. 307944938
0.1

Figure S3: continued

CL0007: ADP-ribosyl glycohydrolase


Tetrahymena thermophila 118396904
94
Acidovorax sp. 365090789
85
Phytophthora sojae 348686615
78
Chlorella variabilis 307103051
51
Beggiatoa sp. PS 153869450
Pbi Can18-4 212R.1
99
Pbi CVM-1 222R.1
Pbi CVB-1 221R.1
82
Pbi NW665.2 189R.1
94
Pbi FR483 Ref N170R
Pbi CVA-1 205R.1
Pbi CVR-1 210R.1
26
SAG Canal-1 898L.1
61
Tetrahymena thermophila 118383037
Tetrahymena thermophila 118369731
99
Tetrahymena thermophila 118396375
Cyanothece sp. 219883156
60
Thermovirga lienii 357420460
91
Spirochaeta sp. 325972461
Pelodictyon phaeoclathratiforme 194336067
Chlorobium phaeobacteroides 189500600
98
Prosthecochloris vibrioformis 145218909
Pelodictyon luteolum 78186694
97
91
Rhizobium leguminosarum 209549929
87
Sorangium cellulosum 162451816
Anaeromyxobacter dehalogenans 86156860
96
alpha proteobacterium 163795751
14
Corynebacterium glucuronalyticum 227488376
Corynebacterium amycolatum 213966178
96
Corynebacterium matruchotii 225021729
100
Megavirus courdo7 371944025
79
Megavirus chiliensis 363540379
Moumouvirus Monve 371944755
100
Acanthamoeba castellanii mamavirus 351737705
93
Acanthamoeba polyphaga mimivirus 311977938
0.2

CL0875: methyltransferase

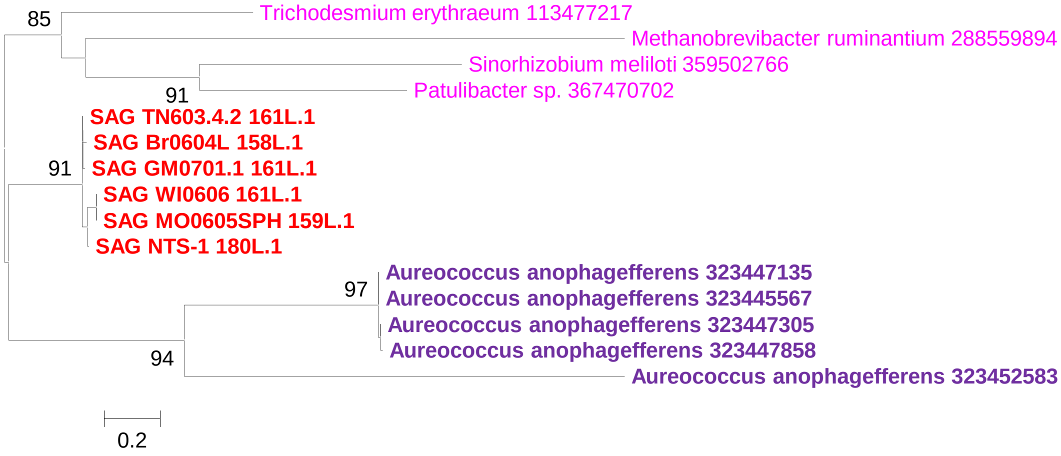
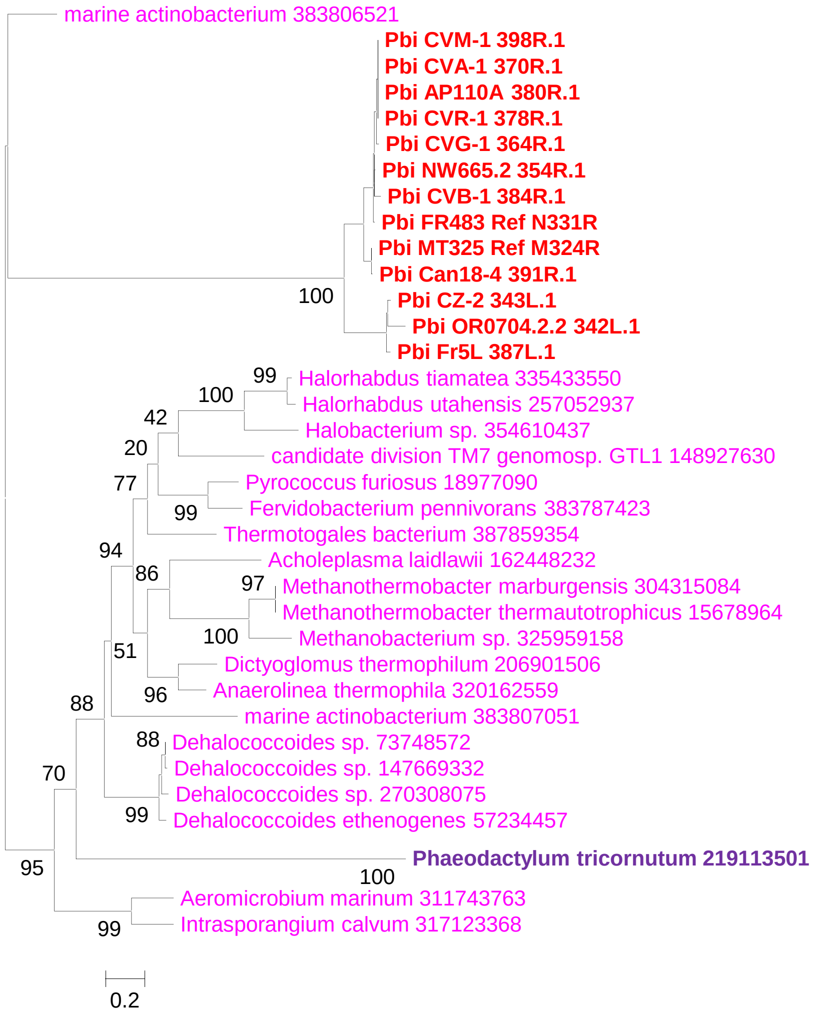

85
Trichodesmium erythraeum 113477217
Methanobrevibacter ruminantium 288559894
Sinorhizobium meliloti 359502766
91
Patulibacter sp. 367470702
SAG TN603.4.2 161L.1
SAG Br0604L 158L.1
91
SAG GM0701.1 161L.1
SAG WI0606 161L.1
SAG MO0605SPH 159L.1
SAG NTS-1 180L.1
97
Aureococcus anophagefferens 323447135
Aureococcus anophagefferens 323445567
Aureococcus anophagefferens 323447305
94
Aureococcus anophagefferens 323447858
Aureococcus anophagefferens 323452583
0.2

Figure S3: continued

CL0489: unknown function


marine actinobacterium 383806521
Pbi CVM-1 398R.1
Pbi CVA-1 370R.1
Pbi AP110A 380R.1
Pbi CVR-1 378R.1
Pbi CVG-1 364R.1
Pbi NW665.2 354R.1
Pbi CVB-1 384R.1
Pbi FR483 Ref N331R
Pbi MT325 Ref M324R
Pbi Can18-4 391R.1
100
Pbi CZ-2 343L.1
Pbi OR0704.2.2 342L.1
Pbi Fr5L 387L.1
99
Halorhabdus tiamatea 335433550
100
Halorhabdus utahensis 257052937
42
Halobacterium sp. 354610437
20
candidate division TM7 genomosp. GTL1 148927630
77
Pyrococcus furiosus 18977090
99
Fervidobacterium pennivorans 383787423
Thermotogales bacterium 387859354
94
Acholeplasma laidlawii 162448232
86
97
Methanothermobacter marburgensis 304315084
Methanothermobacter thermautotrophicus 15678964
100
Methanobacterium sp. 325959158
51
Dictyoglomus thermophilum 206901506
96
Anaerolinea thermophila 320162559
88
marine actinobacterium 383807051
88
Dehalococcoides sp. 73748572
Dehalococcoides sp. 147669332
70
Dehalococcoides sp. 270308075
99
Dehalococcoides ethenogenes 57234457
Phaeodactylum tricornutum 219113501
95
100
Aeromicrobium marinum 311743763
Intrasporangium calvum 317123368
99
0.2

CL0963: glycosyl hydrolase

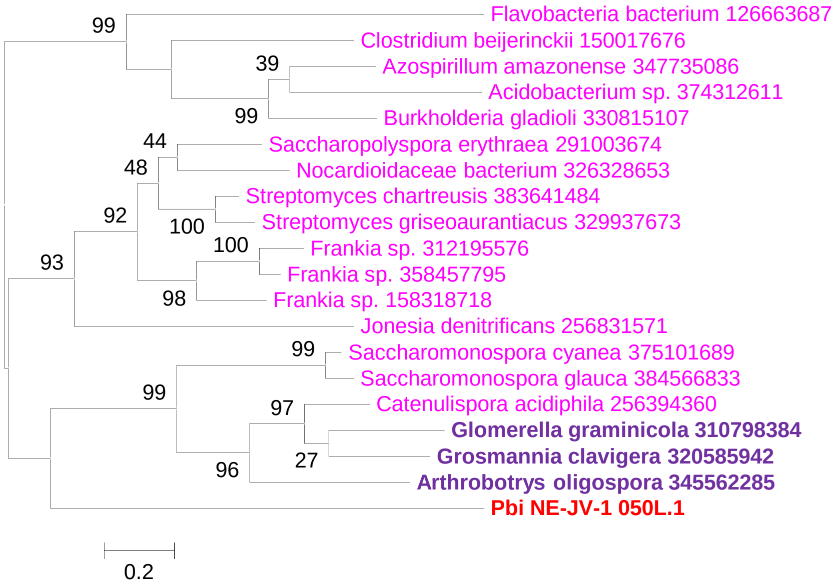
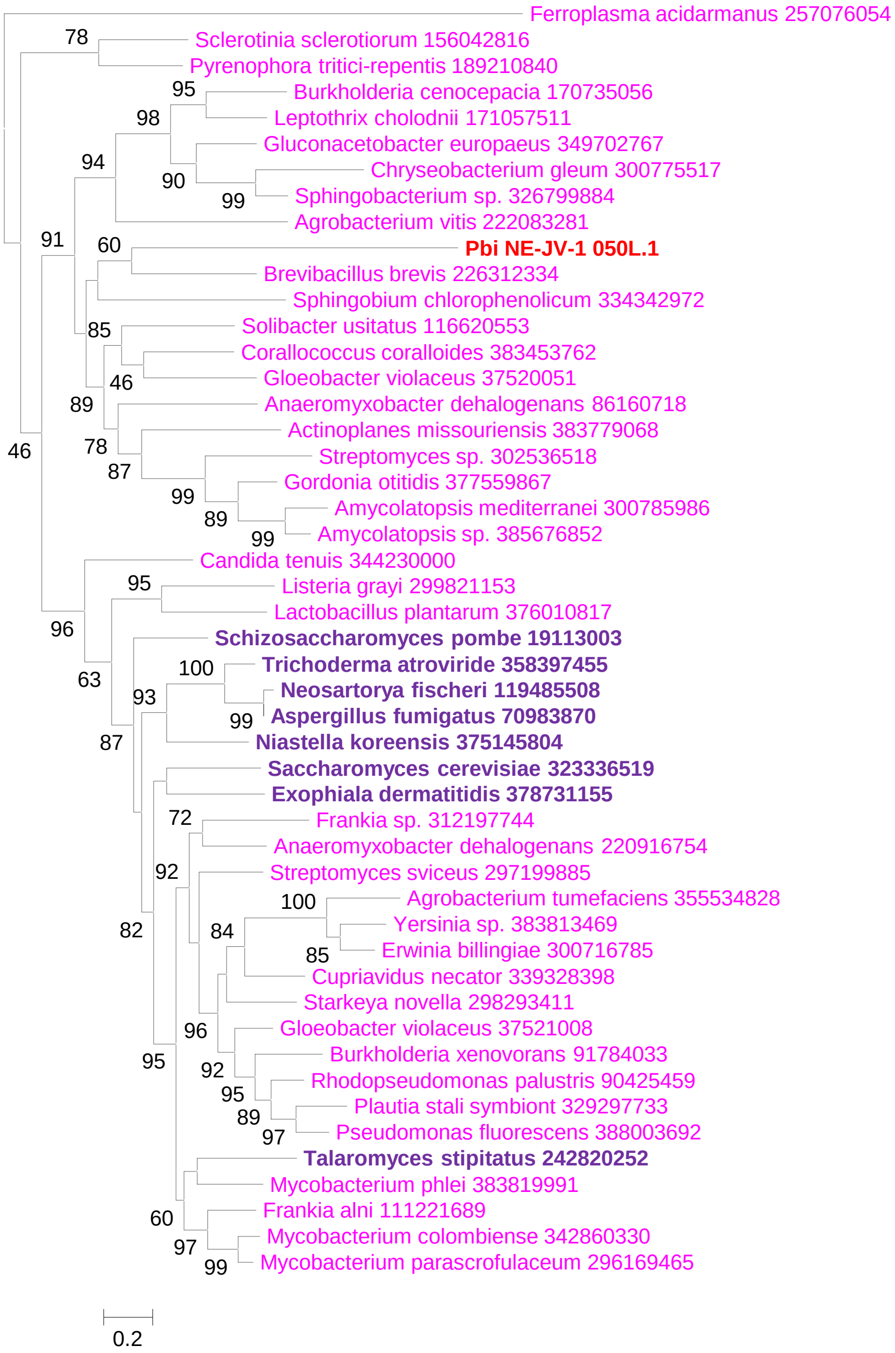

99
Flavobacteria bacterium 126663687
Clostridium beijerinckii 150017676
39
Azospirillum amazonense 347735086
Acidobacterium sp. 374312611
99
Burkholderia gladioli 330815107
44
Saccharopolyspora erythraea 291003674
48
Nocardioidaceae bacterium 326328653
Streptomyces chartreusis 383641484
92
100
Streptomyces griseoaurantiacus 329937673
100
Frankia sp. 312195576
93
Frankia sp. 358457795
98
Frankia sp. 158318718
Jonesia denitrificans 256831571
99
Saccharomonospora cyanea 375101689
Saccharomonospora glauca 384566833
99
97
Catenulispora acidiphila 256394360
Glomerella graminicola 310798384
27
Grosmannia clavigera 320585942
96
Arthrobotrys oligospora 345562285
Pbi NE-JV-1 050L.1
0.2

Figure S3: continued

CL0957: NADH-dependent oxidoreductase


Ferroplasma acidarmanus 257076054
78
Sclerotinia sclerotiorum 156042816
Pyrenophora tritici-repentis 189210840
95
Burkholderia cenocepacia 170735056
98
Leptothrix cholodnii 171057511
Gluconacetobacter europaeus 349702767
94
90
Chryseobacterium gleum 300775517
99
Sphingobacterium sp. 326799884
Agrobacterium vitis 222083281
91
60
Pbi NE-JV-1 050L.1
Brevibacillus brevis 226312334
Sphingobium chlorophenolicum 334342972
85
Solibacter usitatus 116620553
Corallococcus coralloides 383453762
46
Gloeobacter violaceus 37520051
89
Anaeromyxobacter dehalogenans 86160718
Actinoplanes missouriensis 383779068
46
78
Streptomyces sp. 302536518
87
Gordonia otitidis 377559867
99
89
Amycolatopsis mediterranei 300785986
99
Amycolatopsis sp. 385676852
Candida tenuis 344230000
95
Listeria grayi 299821153
Lactobacillus plantarum 376010817
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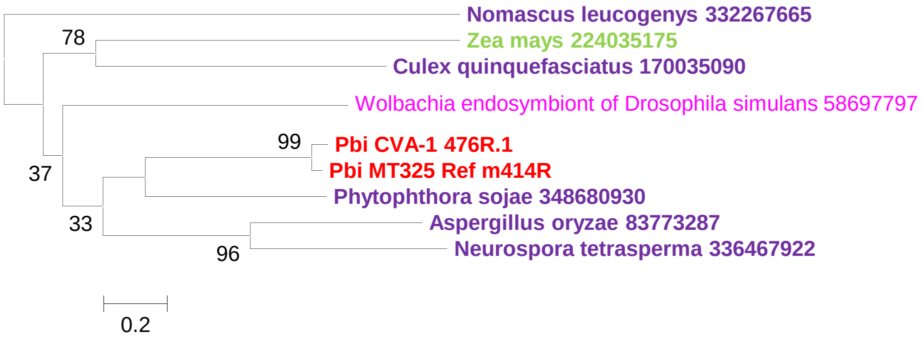
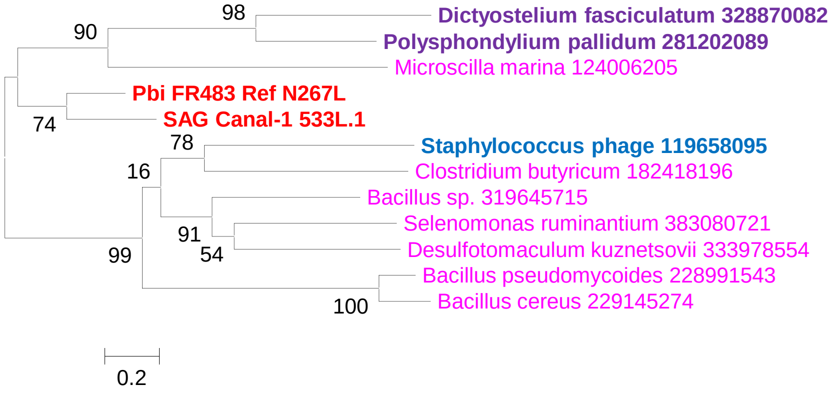
Schizosaccharomyces pombe 19113003
100
Trichoderma atroviride 358397455
63
93
Neosartorya fischeri 119485508
99
Aspergillus fumigatus 70983870
87
Niastella koreensis 375145804
Saccharomyces cerevisiae 323336519
Exophiala dermatitidis 378731155
72
Frankia sp. 312197744
Anaeromyxobacter dehalogenans 220916754
92
Streptomyces sviceus 297199885
100
Agrobacterium tumefaciens 355534828
82
84
Yersinia sp. 383813469
85
Erwinia billingiae 300716785
Cupriavidus necator 339328398
Starkeya novella 298293411
96
Gloeobacter violaceus 37521008
95
Burkholderia xenovorans 91784033
92
Rhodopseudomonas palustris 90425459
95
Plautia stali symbiont 329297733
89
97
Pseudomonas fluorescens 388003692
Talaromyces stipitatus 242820252
Mycobacterium phlei 383819991
Frankia alni 111221689
60
97
Mycobacterium colombiense 342860330
99
Mycobacterium parascrofulaceum 296169465
0.2

Figure S3: continued

CL0011: unkwown function


Nomascus leucogenys 332267665
78
Zea mays 224035175
Culex quinquefasciatus 170035090
Wolbachia endosymbiont of Drosophila simulans 58697797
99
Pbi CVA-1 476R.1
37
Pbi MT325 Ref m414R
Phytophthora sojae 348680930
33
Aspergillus oryzae 83773287
96
Neurospora tetrasperma 336467922
0.2

CL0056: unknown function


98
Dictyostelium fasciculatum 328870082
90
Polysphondylium pallidum 281202089
Microscilla marina 124006205
Pbi FR483 Ref N267L
74
SAG Canal-1 533L.1
78
Staphylococcus phage 119658095
16
Clostridium butyricum 182418196
Bacillus sp. 319645715
91
Selenomonas ruminantium 383080721
99
54
Desulfotomaculum kuznetsovii 333978554
Bacillus pseudomycoides 228991543
100
Bacillus cereus 229145274
0.2

CL0503: unknown function

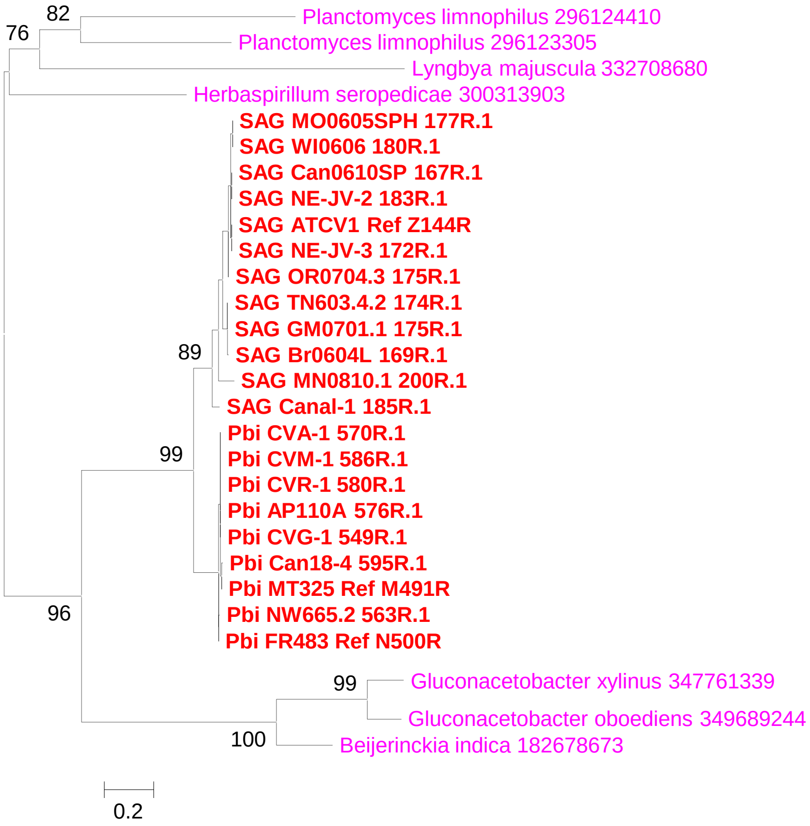
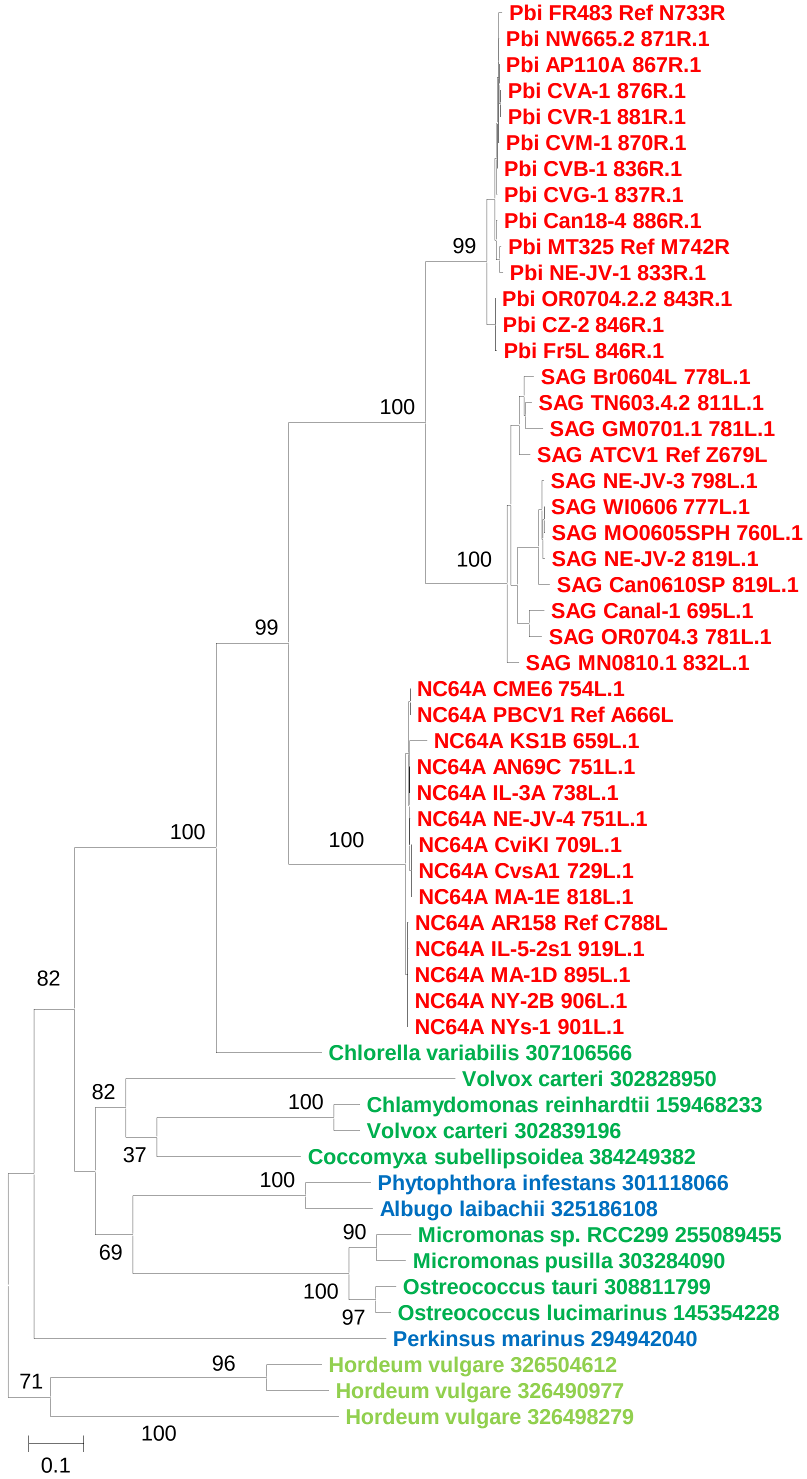


Figure S3: continued

Table S4: sister groups to non-ancestral CV proteins based on 35 phylogenetic trees shown in Figure S3

Cluster ID	Sister group	Function
CL0007	**Tetrahymena thermophila (Alveolata)**	ADP-ribosyl glycohydrolase
CL0011	**Phytophthora sojae (stramenopiles)**	unknown
CL0037	**Bacteria**	beta-lactamase
CL0049	**Chlorophyta**	NADH-dependent fumarate reductase
CL0055	**Bacteria**	unknown
CL0056	Mixed eukaryotes and bacteria	unknown
CL0063	**Plantae**	unknown
CL0065	**Bacteria**	unknown
CL0222	**Bacteria**	DNA methylase
CL0356	**Oceanicola granulosus (Bacteria)**	methyltransferase
CL0375	**Coccomyxa subellipsoidea (Chlorophyta)**	glycosyltransferase
CL0466	**Chlorophyta**	MIP family channel protein
CL0482	**Bacteria**	glutaredoxin
CL0489	**Actinobacterium (Bacteria)**	unknown
CL0503	**Bacteria**	unknown
CL0533	Basal	unknown
CL0561	**Polynucleobacter necessarius (Bacteria)**	Glycosyltransferase family 17
CL0607	**Waddlia chondrophila (Bacteria)**	methyltransferase
CL0624	**Chlorophyta**	THYLAKOID FORMATION 1; inositol phosphatase-like protein
CL0739	Basal	aspartate carbamoyltransferase
CL0767	**Aureococcus anophagefferens (diatom)**	unknown
CL0778	**Viridiplantae**	unknown
CL0780	**Chlorella (Chlorophyta)**	dUDP-D-glucose 4,6-dehydratase
CL0787	**Viridiplantae**	Potassium transporter
CL0792	**Kytococcus sedentarius (Bacteria)**	mannose-6-phosphate isomerase
CL0796	**Chlorophyta**	ribonucleoside-triphosphate reductase
CL0875	Basal	methyltransferase
CL0876	**Brevundimonas diminuta (Bacteria)**	methyltransferase
CL0878	**Prochlorococcus marinus (Bacteria)**	dTDP-glucose pyrophosphorylase/HAD superfamily hydrolase
CL0879	**Prochlorococcus marinus (Bacteria)**	unknown
CL0940	**Actinobacterium (Bacteria)**	Nitroreductase
CL0957	**Brevibacillus brevis (Bacteria)**	NADH-dependent oxidoreductase
CL0963	Mixed eukaryotes and bacteria	glycosyl hydrolase
CL0978	**Streptophyta**	unknown
CL0989	**Bacteria**	unknown

Figure S4



CL0450: translation elongation factor ElF3

CL0511: unknown function

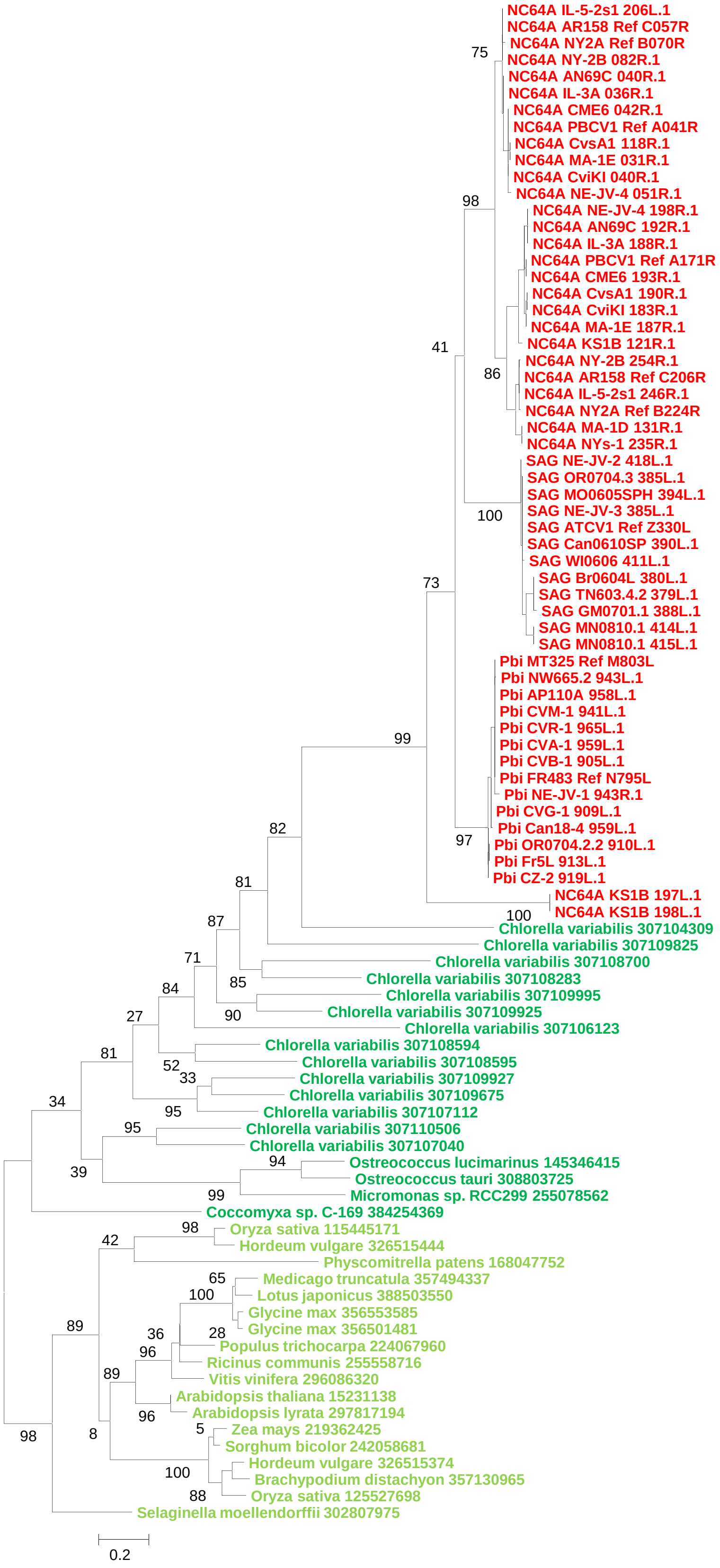
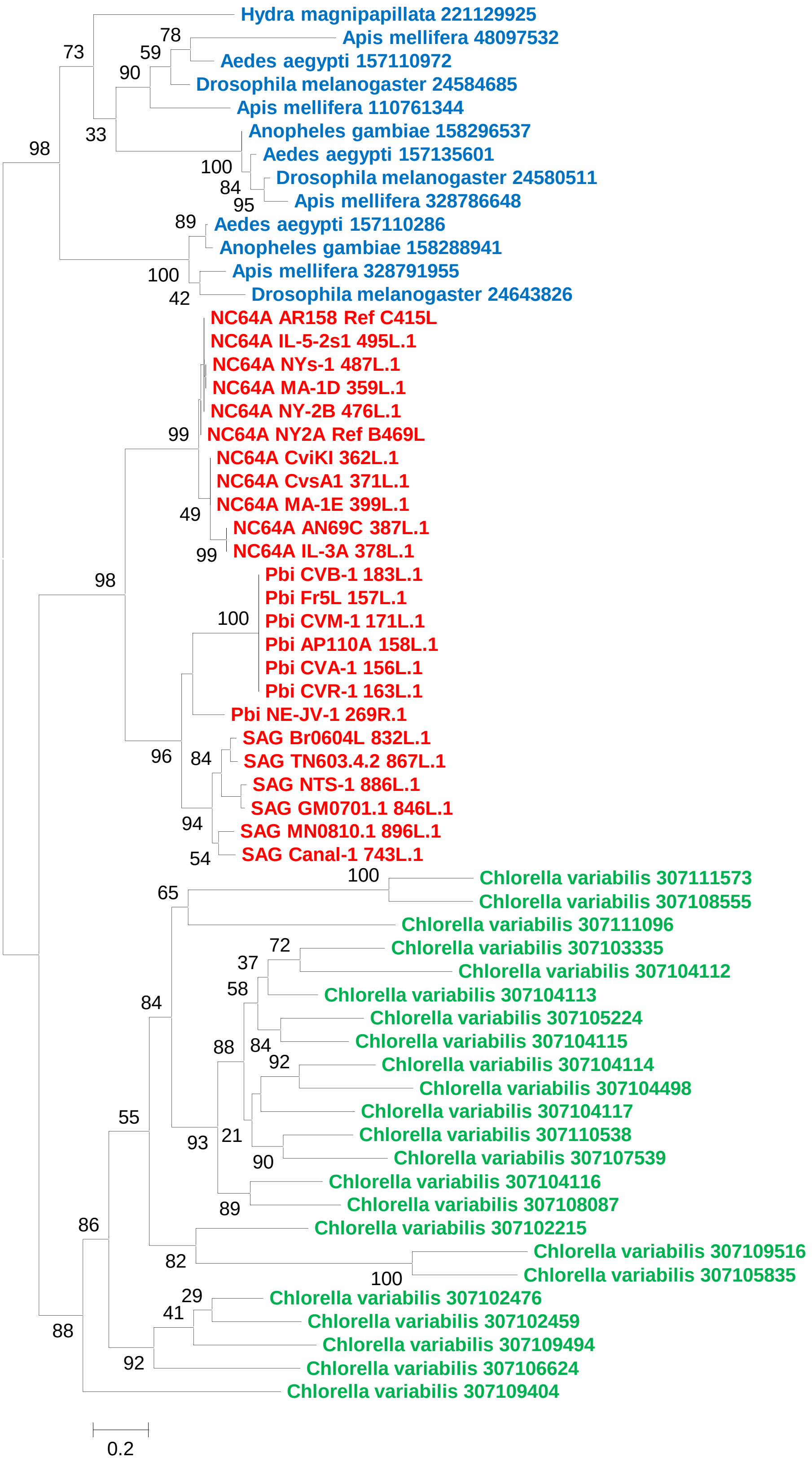


Figure S4: continued

CL0288: chitin deacetylase


Hydra magnipapillata 221129925
Apis mellifera 48097532
Aedes aegypti 157110972
Drosophila melanogaster 24584685
Apis mellifera 110761344
Anopheles gambiae 158296537
Aedes aegypti 157135601
Drosophila melanogaster 24580511
Apis mellifera 328786648
Aedes aegypti 157110286
Anopheles gambiae 158288941
Apis mellifera 328791955
Drosophila melanogaster 24643826
NC64A AR158 Ref C415L
NC64A IL-5-2s1 495L.1
NC64A NYs-1 487L.1
NC64A MA-1D 359L.1
NC64A NY-2B 476L.1
NC64A NY2A Ref B469L
NC64A CviKI 362L.1
NC64A CvsA1 371L.1
NC64A MA-1E 399L.1
NC64A AN69C 387L.1
NC64A IL-3A 378L.1
Pbi CVB-1 183L.1
Pbi Fr5L 157L.1
Pbi CVM-1 171L.1
Pbi AP110A 158L.1
Pbi CVA-1 156L.1
Pbi CVR-1 163L.1
Pbi NE-JV-1 269R.1
SAG Br0604L 832L.1
SAG TN603.4.2 867L.1
SAG NTS-1 886L.1
SAG GM0701.1 846L.1
SAG MN0810.1 896L.1
SAG Canal-1 743L.1
Chlorella variabilis 307111573
Chlorella variabilis 307108555
Chlorella variabilis 307111096
Chlorella variabilis 307103335
Chlorella variabilis 307104112
Chlorella variabilis 307104113
Chlorella variabilis 307105224
Chlorella variabilis 307104115
Chlorella variabilis 307104114
Chlorella variabilis 307104498
Chlorella variabilis 307104117
Chlorella variabilis 307110538
Chlorella variabilis 307107539
Chlorella variabilis 307104116
Chlorella variabilis 307108087
Chlorella variabilis 307102215
Chlorella variabilis 307109516
Chlorella variabilis 307105835
Chlorella variabilis 307102476
Chlorella variabilis 307102459
Chlorella variabilis 307109494
Chlorella variabilis 307106624
Chlorella variabilis 307109404
78
59
73
90
33
98
100
84
95
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42
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98
100
96
84
94
54
100
65
72
37
58
84
88
84
92
55
93
21
90
89
86
82
100
29
41
88
92
0.2

Figure S4: continued

CN0022: unknown function

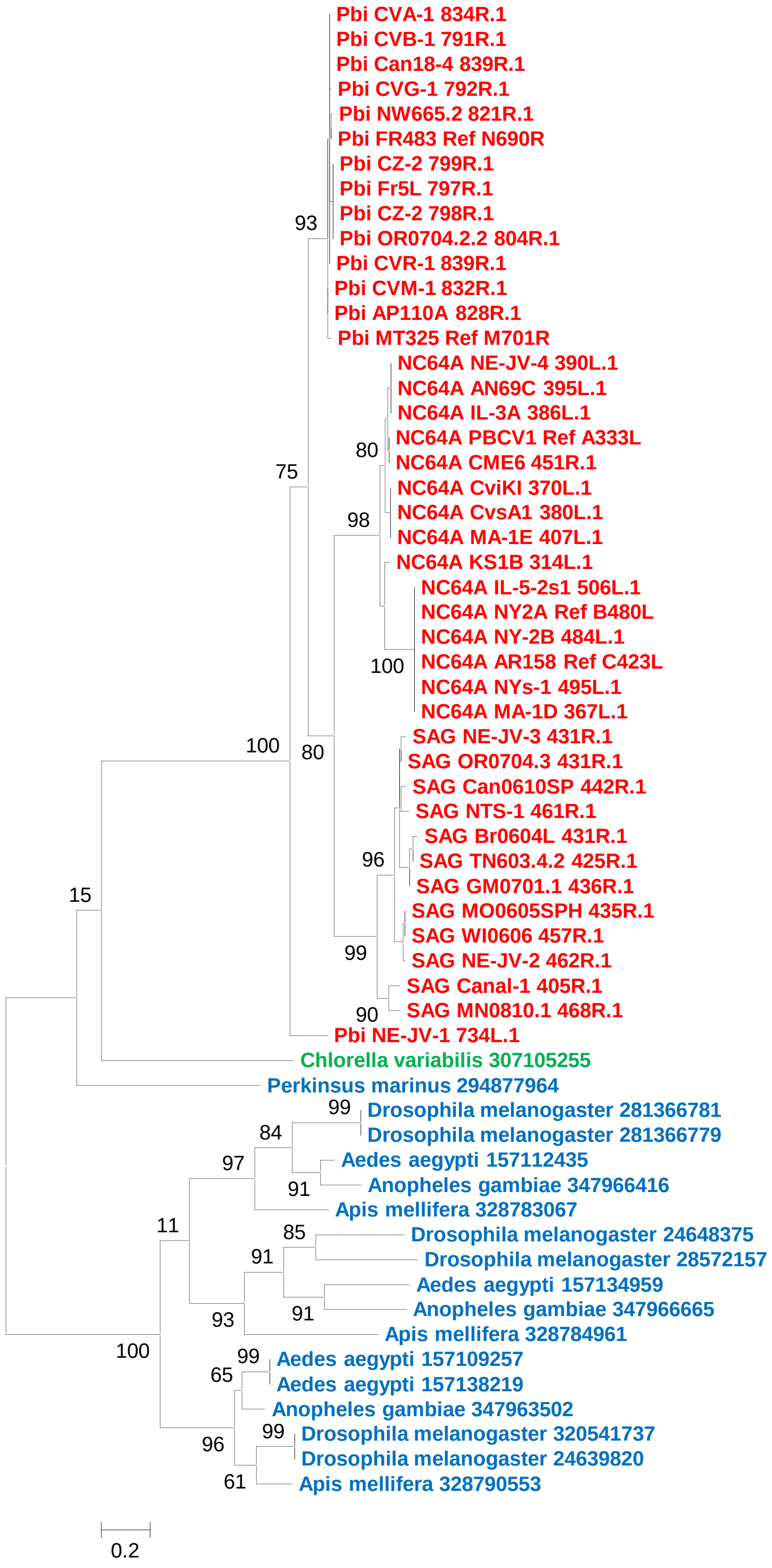


Figure S4: continued

CL0887: chitinase

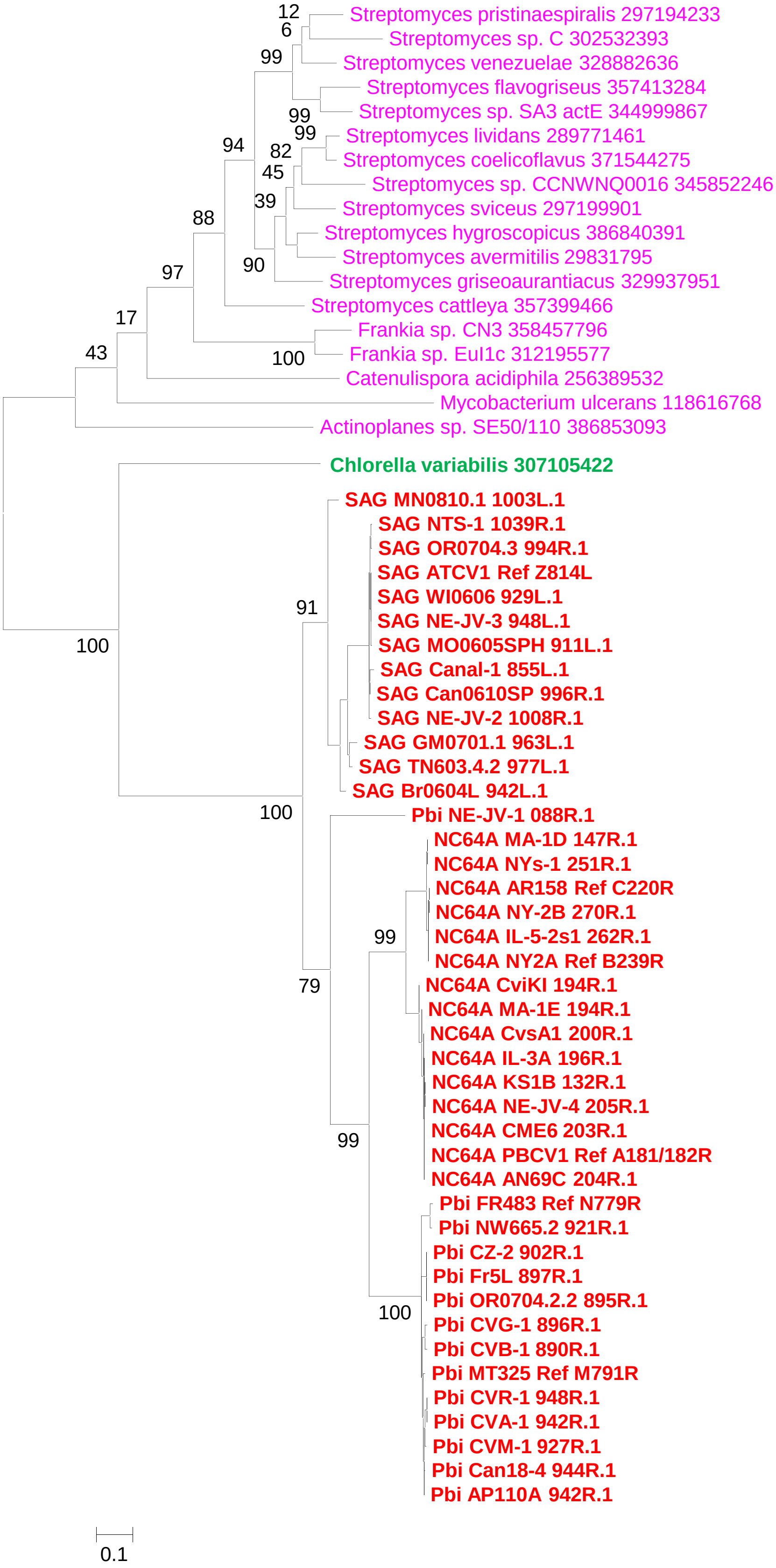


Figure S4: continued

CL0531: unknown function

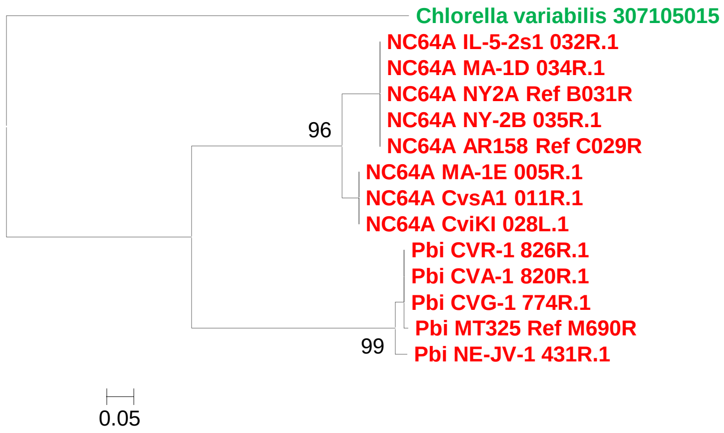


Figure S4: continued

Table S5: attributes of the sequenced chloroviruses

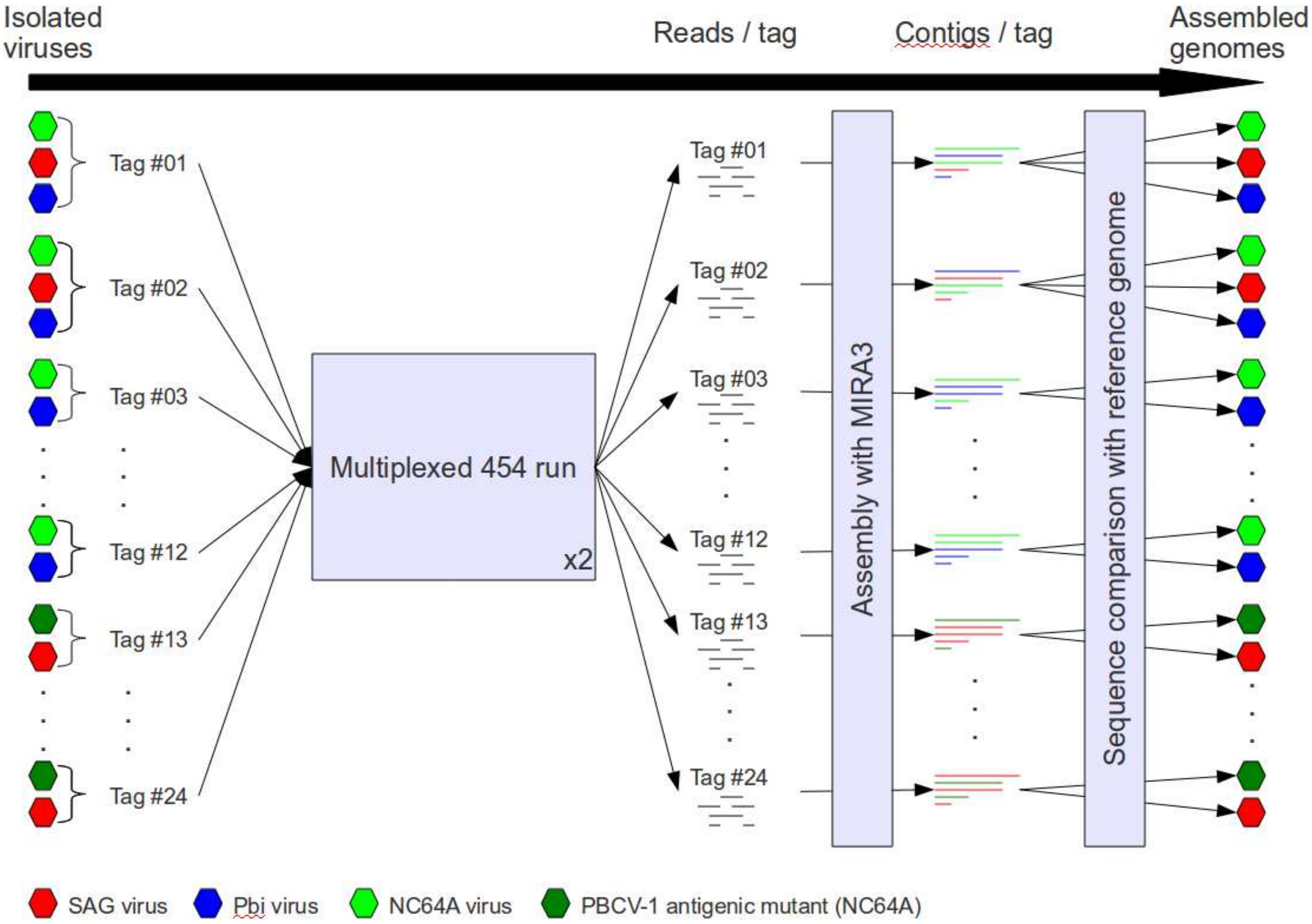
Virus	Host	Attributes and comments (plaque size, plaque morphology, gene content, etc.)	Source of isolate	Date collected
Chlorella variabilis NC64A Virus Isolates				
CviKI	Chlorella NC64A	from Yamada Lab, Japan; encodes Hyaluronan Synthetase and Chitin Synthase	Kyoto, Japan	1990
IL-3A	Chlorella NC64A	Serves as the "null mutant" in the hyaluronan/chitin competition series	IL, USA	Oct. 1983
CvsA1	Chlorella NC64A	from Yamada Lab, Japan; encodes 2 Chitin Synthase genes (& gfat)	Sawara, Japan	April 1992
MA-1D	Chlorella NC64A	see [1] table 2; Small plaque-forming virus	MA, USA	Aug. 1984
NYs-1	Chlorella NC64A	see [1] table 2; Small plaque-forming virus	NY, USA (river)	Aug. 1985
IL-5-2s1	Chlorella NC64A	see [1] table 2; Small plaque-forming virus	IL, USA (farm pond)	May 1986
KS1B	Chlorella NC64A	Small plaque-forming virus	Kansas, USA	May 2003
NY-2B	Chlorella NC64A	see [1] table 2; Very small plaque-forming virus	NY, USA (river)	Aug. 1984
AN69C	Chlorella NC64A	Small plaque-forming virus	Canberra, Australia	March 1995
MA-1E	Chlorella NC64A	see [1] table 2; Gene re-arrangement and insertion in the PBCV-1_A250R-homolog locus	MA, USA	Aug 1984
NE-JV4	Chlorella NC64A	regular plaques of medium-to-large size	Rowe Bird Sanctuary; NE, USA	May 2008
Chlorella heliozoae SAG 3.83 Virus Isolates				
NTS-1	Chlorella SAG3.83	Alkaline lake isolates, fuzzy plaques	Next to Smith Lake, NE, USA (CLNWR)	June 2008
Canal-1	Chlorella SAG3.83	Alkaline lake isolates, fuzzy plaques; does not completely lyse a culture	canal exiting Smith Lake, NE, USA (CLNWR)	June 2008
TN603.4.2	Chlorella SAG3.83	First SAG 3.83 virus found in the USA; Large, clear plaques;	Tennessee, USA	April 2006
WI0606	Chlorella SAG3.83	Normal plaque size and shape; Geographic site	Madison Wisconsin, USA	July 2006
Br0604L	Chlorella SAG3.83	Normal plaque size and shape; Geographic site	St. Paul, Brazil	2006
GM0701.1	Chlorella SAG3.83	Normal plaque size and shape; Geographic site	Guatemala	January 2007
MO0605SPH	Chlorella SAG3.83	Cloudy plaques; Geographic site	Missouri, USA	2006
Can0610SP	Chlorella SAG3.83	Normal plaque size and shape; Geographic site	British Columbia, Canada	August 2006
OR0704.3	Chlorella SAG3.83	Normal plaque size and shape; Geographic site	Willamette River, Corvallis, Oregon, USA	July 2007
MN0810.1	Chlorella SAG3.83	Normal plaque size and shape; Geographic site (abandoned mine)	Minnesota, USA	August 2008
NE-JV2	Chlorella SAG3.83	small, irregularly shaped plaques with fuzzy edges	Rowe Bird Sanctuary; Gibbon, NE, USA	May 2008
NE-JV3	Chlorella SAG3.83	medium sized, irregularly shaped plaques with fuzzy edges	Gudmundsen Ranch	May 2008

Virus	Host	Attributes and comments (plaque size, plaque morphology, gene content, etc.)	Source of isolate	Date collected
Micractinium conductrix Pbi Virus Isolates				
CVA-1	Micractinium Pbi	see [2]; Normal plaque size and shape	Amönau, Germany	1984
CVB-1	Micractinium Pbi	see [2]; Normal plaque size and shape	Berlin, Germany	1984
CVG-1	Micractinium Pbi	see [2]; Normal plaque size and shape	Göttingen, Germany	1984
CVM-1	Micractinium Pbi	see [2]; Normal plaque size and shape	Marburg, Germany	1984
CVR-1	Micractinium Pbi	see [2]; Normal plaque size and shape	Rauschenberg, Germany	1984
NW665.2	Micractinium Pbi	Small, regularly shaped plaques	Norway	1995
AP110A	Micractinium Pbi	High plaque numbers; 1 of a collection of 10 "AP" viruses	unknown	unknown
Can18-4	Micractinium Pbi	Normal plaque size and shape; Geographic site	Canada	1995
CZ-2	Micractinium Pbi	Normal plaque size and shape; Geographic site	Czech Republic	1995
Fr5L	Micractinium Pbi	Normal plaque size and shape; Geographic site	France	1995
OR0704.2.2	Micractinium Pbi	Normal plaque size and shape; Geographic site	Willamette River, Corvallis, Oregon	July 2007
NE-JV1	Micractinium Pbi	Small, regularly shaped plaques with fuzzy edges	South Platte River, Nebraska	May 2008

1. Van Etten JL, Lane LC, Meints RH: **Viruses and virus-like particles of eukaryotic algae**. *Microbiol. Rev.* 1991, **55**:586–620.

2. Reisser W, Burbank D, Meints R, Becker B, Van Etten J: **Viruses distinguish symbiotic Chlorella spp. of Paramecium bursaria**. *Endocytobiosis and Cell Research* 1991, **7**:247–251.

Figure S5


Isolated viruses
Reads / tag
Contigs / tag
Assembled genomes
Tag #01
Tag #02
Tag #03
Tag #12
Tag #13
Tag #24
Multiplexed 454 run
x2
Tag #01
Tag #02
Tag #03
Tag #12
Tag #13
Tag #24
Assembly with MIRA3
Sequence comparison with reference genome
SAG virus
Pbi virus
NC64A virus
PBCV-1 antigenic mutant (NC64A)