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Comparative Genomics of Chrysochromulina Ericina Virus and Other Microalga-Infecting Large DNA Viruses Highlights Their Intricate Evolutionary Relationship with the Established *Mimiviridae* Family

Lucie Gallot-Lavallée,[a] Guillaume Blanc,[a]* Jean-Michel Claverie[a,b]

Information Génomique et Structurale, UMR 7256 (IMM FR 3479) Centre National de la Recherche Scientifique & Aix-Marseille University, Marseille, France[a]; Assistance Publique des Hôpitaux de Marseille, La Timone, Marseille, France[b]

ABSTRACT Chrysochromulina ericina virus CeV-01B (CeV) was isolated from Norwegian coastal waters in 1998. Its icosahedral particle is 160 nm in diameter and encloses a 474-kb double-stranded DNA (dsDNA) genome. This virus, although infecting a microalga (the haptophyceae *Haptolina ericina*, formerly *Chrysochromulina ericina*), is phylogenetically related to members of the *Mimiviridae* family, initially established with the acanthamoeba-infecting mimivirus and megavirus as prototypes. This family was later split into two genera (*Mimivirus* and *Cafeteriavirus*) following the characterization of a virus infecting the heterotrophic stramenopile *Cafeteria roenbergensis* (CroV). CeV, as well as two of its close relatives, which infect the unicellular photosynthetic eukaryotes *Phaeocystis globosa* (Phaeocystis globosa virus [PgV]) and *Aureococcus anophagefferens* (Aureococcus anophagefferens virus [AaV]), are currently unclassified by the International Committee on Viral Taxonomy (ICTV). The detailed comparative analysis of the CeV genome presented here confirms the phylogenetic affinity of this emerging group of microalga-infecting viruses with the *Mimiviridae* but argues in favor of their classification inside a distinct clade within the family. Although CeV, PgV, and AaV share more common features among them than with the larger *Mimiviridae*, they also exhibit a large complement of unique genes, attesting to their complex evolutionary history. We identified several gene fusion events and cases of convergent evolution involving independent lateral gene acquisitions. Finally, CeV possesses an unusual number of inteins, some of which are closely related despite being inserted in nonhomologous genes. This appears to contradict the paradigm of allele-specific inteins and suggests that the *Mimiviridae* are especially efficient in spreading inteins while enlarging their repertoire of homing genes.

IMPORTANCE Although it infects the microalga *Chrysochromulina ericina*, CeV is more closely related to acanthamoeba-infecting viruses of the *Mimiviridae* family than to any member of the *Phycodnaviridae*, the ICTV-approved family historically including all alga-infecting large dsDNA viruses. CeV, as well as its relatives that infect the microalgae *Phaeocystic globosa* (PgV) and *Aureococcus anophagefferens* (AaV), remains officially unclassified and a source of confusion in the literature. Our comparative analysis of the CeV genome in the context of this emerging group of alga-infecting viruses suggests that they belong to a distinct clade within the established *Mimiviridae* family. The presence of a large number of unique genes as well as specific gene fusion events, evolutionary convergences, and inteins integrated at unusual locations document the complex evolutionary history of the CeV lineage.

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Address correspondence to Lucie Gallot-Lavallée, Lucie.Gallot-Lavallee@igs.cnrs-mrs.fr, or Jean-Michel Claverie, Jean-Michel.Claverie@univ-amu.fr.

* Present address: Guillaume Blanc, Mediterranean Institute of Oceanography (MIO), Aix Marseille Université, Université de Toulon, CNRS/INSU, IRD, UM 110, Marseille, France.

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KEYWORDS Aureococcus anophagefferens virus, Chrysochromulina ericina virus, Haptolina ericina virus, Megamimivirinae, Mesomimivirinae, *Mimiviridae*, nucleocytoplasmic virus, Phaeocystis globosa virus

Several new viral families have been recently created (or proposed) following the discovery of highly diverse double-stranded DNA (dsDNA) giant viruses (initially defined as those with particles visible under a light microscope), all of which exhibit large genomes (>300 kb) and infect unicellular eukaryotes (reviewed in references 1–3). Among those new families, the most populated is the *Mimiviridae*, and it is the only one officially recognized by the International Committee on Viral Taxonomy (ICTV). The *Mimiviridae* comprises two registered genera: the *Mimivirus* and the *Cafeteriavirus*. The *Mimivirus* genus includes several dozen members distributed among three clades (A, B, and C), all of which infect *Acanthamoeba* and have pseudoicosahedral particles approximately 700 nm in diameter and genomes of about one megabase in length (4). The genus *Cafeteriavirus* contains a single member, *Cafeteria roenbergensis virus* (CroV). This virus is markedly different from the mimiviruses, having smaller particles (300 nm in diameter) and a smaller, 730-kb genome, with its host being the heterotrophic stramenopile *C. roenbergensis* (5). Prior to defining these two genera, metagenomic studies had already hinted at the presence of mimivirus relatives in marine environments (6). The successful isolation and characterization of several of these viruses showed that they correspond to smaller icosahedral particles (140 to 180 nm in diameter) packing smaller dsDNA genomes (370 to 475 kb in length) (7–9). In core gene phylogenies, these viruses clearly cluster with the *Mimiviridae*, although they appear to constitute a distinct clade (Fig. 1) (1, 2, 7, 8). This clade comprises only viruses infecting photosynthetic hosts (i.e., microalgae) from different taxa: haptophyceae for Phaeocystis globosa virus (PgV) and Haptolina ericina virus and stramenopile for Aureococcus anophagefferens virus (AaV). This emerging subgroup within the *Mimiviridae* appears to include other lesser characterized members, such as Phaeocystis pouchetii virus (PpV), Prymnesium kappa virus (PkV) (10), and Pyramimonas orientalis virus (PoV). It also includes a number of other nonisolated candidates predicted solely from the assembly of metagenomics sequence data (11), such as the Organic Lake phycodnaviruses (OLPV1 and -2) (12). Since these viruses infect algae, a paraphyletic group of organisms, they were originally classified as members of the *Phycodnaviridae*, although this historical family increasingly encompasses viruses with little phylogenetic relationship (13, 14). One of the goals of the present study is to clarify this issue.

Aside from their gene-based phylogenetic clustering within the *Mimiviridae*, the members of this emerging clade exhibit additional features, such as AT-rich genomes encoding full DNA transcription and replication machinery (needed for their intracytoplasmic replication) (2) and a special version of the MutS mismatch DNA repair protein strangely related to octocorals (15). Most of them also encode an asparagine synthase (AsnS) (11). The *Mimiviridae* family also harbors the only viruses known to allow the replication of virophages (7, 12, 16–18).

Chrysochromulina ericina virus (CeV) was isolated from Norwegian coastal waters in 1998 but was only recently fully sequenced (9). It replicates within the cytoplasm of *H. ericina* (formerly *Chrysochromulina ericina*) with a lytic cycle lasting 14 to 19 h, resulting in the release of thousands of icosahedral particles 160 nm in diameter (19). Its host is distributed worldwide. Here, we performed a detailed comparative analysis of the genome of CeV and of its closest fully sequenced relatives (PgV and AaV) to provide support for their classification within a new clade in the *Mimiviridae*, as well as to investigate their evolutionary relationship with the rest of the family.

RESULTS

CeV genome global analysis. In line with its virion size, the CeV genome (473,558 bp) is larger than those of PgV and AaV (Table 1) and is the largest among those of alga-infecting viruses from any other family. We identified 512 putative protein-coding

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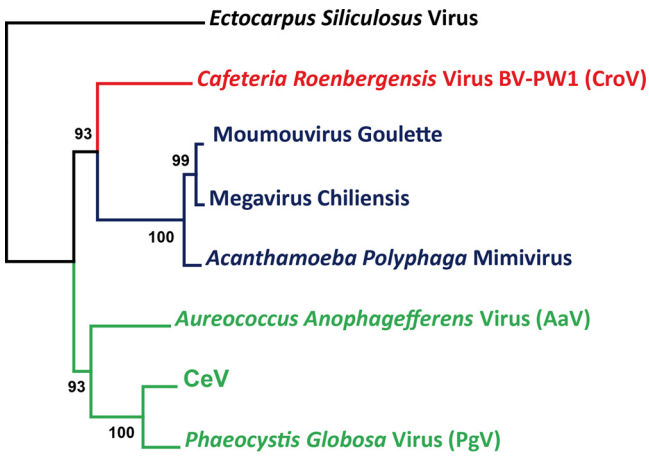

FIG 1 Phylogeny of the concatenation of MCP, DNAPol B, and DNA packaging ATPase. The phylogenetic tree was built using PhyML (58) based on multiple alignments generated using Expresso (60). The tree was rooted with *Ectocarpus siliculosus virus* (*Phaeovirus*). Representatives of the genera *Mimivirus* (blue) and *Cafeteriavirus* (red) have been included, as well as the three fully sequenced alga-infecting *Mimiviridae* relatives (green). The tree was drawn using MEGA7 (61).

genes with an average length of 280 codons (ranging from 41 to 2,317 codons) and 12 tRNA genes (two $tRNA^{Leu}$, two $tRNA^{Ser}$, one $tRNA^{Ala}$, one $tRNA^{Ile}$, two $tRNA^{Lys}$, one $tRNA^{Gln}$, one $tRNA^{Asn}$, one $tRNA^{Arg}$, and one $tRNA^{Gly}$). Intergenic sequences are very short (82.5 nucleotides [nt] on average) and exhibit higher A+T contents than average (85% versus 73.7%), suggesting that the loss of C+G nucleotides is an ongoing evolutionary process only slowed down by the negative selection pressure applied on protein-coding regions (Table 1). Compared to the NCBI nonredundant sequence database NR (including *Mimiviridae*), 43% (218) of the predicted proteins did not exhibit a significant match (E value of $<10^{-5}$ by BLASTP). This proportion of ORFans (i.e., without recognizable homologs within the whole NR database) is similar to that of other alga-infecting *Mimiviridae* (AaV, 45%; OLPV1, 44%; PgV, 43%). Among the 293 predicted proteins with a database homolog, 221 (75.4%) had their best match in eukaryote-infecting large dsDNA viruses, most of which (214/221, i.e., 96.8%) were members of the *Mimiviridae* family, mainly PgV (with 144 best matches) and the two OLPVs (with 30 best matches). The 72 nonviral best matches were distributed between bacteria (30) and eukaryotes (43), including 7 open reading frames (ORFs) in haptophytes (i.e., the taxon of the CeV host), pointing out potential horizontal gene transfers (HGT). A comparison (dot plot) of the orthologous gene positions in the genomes of CeV's closest relatives indicates numerous rearrangements (data not shown).

Phylogenetic analyses. To reconstruct the relationship between the viruses composing the *Mimiviridae* alga-infecting clade, we used a concatenation of the DNA polymerases, ATP DNA-packaging enzymes, and closest orthologs of the major capsid protein (MCP1). Consistent with the distribution of CeV best hits in the NR database, PgV appears to be its closest relative among fully sequenced viruses (Fig. 1). However,

TABLE 1 Genomic features of the *Mimiviridae*

Virus	Genome size (kbp)	No. of ORFs	Avg ORF size (bp)	Genome GC%	Coding density	GC%	
						ORF	Inter-ORF
Megavirus	1,259	1,120	1,015	25.3	0.90	26.5	14.1
Mimivirus	1,181	979	1,080	28.0	0.90	29.1	18.3
CroV	730	544	1,025	23.3	0.76	24.3	20.1
AaV	371	377	870	28.7	0.88	29.9	19.8
OLPV1[a]			697	29.5	0.86	30.2	25.0
PgV	460	434	960	32.0	0.91	33.7	15.3
CeV	474	512	840	25.4	0.91	26.3	15.7

[a]OLPV1 statistics are based on a 344,723-bp-long contig (12).

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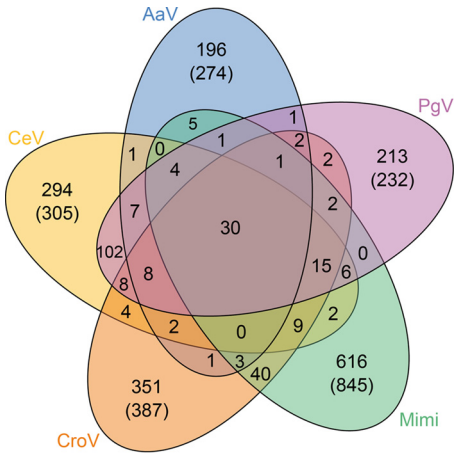

FIG 2 Venn diagram indicating the global proximity in gene content of CeV, its two closest relatives, PgV and AaV, and one member of each genus of the family *Mimiviridae* (*Cafeteriavirus* genus, CroV; *Mimivirus* genus, Mimi). The numbers in parentheses correspond to the raw number of encoded proteins without a homolog in the four other viruses. The numbers without parentheses indicate how many distinct clusters they constitute. The analysis was driven using OrthoMCL software (20), with a 10^{-5} E-value threshold and 1.5-mcl inflation parameter.

when we included OLPV1 in the analysis, no consistent pattern was found for the branching order of CeV, PgV, and OLPV after they diverged from their common ancestor with AaV. Moreover, applying the Shimodaira and Hasegawa (SH) test (see Materials and Methods) to each of the 39 groups of orthologous proteins shared by the 4 viruses (CeV, PgV, OLPV, and AaV) did not produce a conclusive answer. Such ambiguous results could be due to divergence times too close to be resolved, orthologous gene exchanges among these viruses, and/or compositional constraint similarities blurring the phylogenetic signal.

Gene content. Using OrthoMCL (20), we analyzed the groups of orthologues shared by mimivirus, CroV, and the three fully sequenced alga-infecting viruses, CeV, PgV, and AaV (Fig. 2). A striking result is that among the hundreds of proteins encoded by these clearly related viruses, only 30 are shared by all of them (a number dropping down to 19 when including Moumouvirus and Megavirus). Thus, including the new clade of alga-infecting viruses in the *Mimiviridae* causes the extended family to rest on an amazingly small proportion of common core genes. On the other hand, each viral genome exhibits a high number of unique genes (i.e., without recognizable homologs in the other *Mimiviridae*) (305 for CeV, 274 for AaV, 232 for PgV, 387 for CroV, and 845 for mimivirus) (Fig. 2). As 68% of CeV-unique genes correspond to ORFans, postulating a high frequency of horizontal exchanges with known viruses or cellular organisms is clearly not sufficient to explain their origin. Finally, some paralogs are sporadically present in the various viruses. Altogether, these large differences in the gene contents of these various viruses, which nevertheless share a strong phylogenetic signal of common ancestry, are rather puzzling and at least suggest a complex evolutionary history of the family.

Unique features common to the alga-infecting *Mimiviridae*. We further investigated if the alga-infecting viruses possessed common genes/functions not shared with the other *Mimiviridae*. As PgV, CeV, and AaV infect photosynthetic protozoans (at variance with the other *Mimiviridae*), we postulated that genomic features exclusive to them could be linked to this specific lifestyle. Only 7 of such shared genes were identified, among which a single one corresponds to a predicted function: an ERCC4-type DNA repair nuclease (YP_009173624.1, or CeV_369). Such enzymes are usually part of the cellular response against UV-induced DNA damage (21, 22), also known to occur in eukaryotic viruses (23). While infecting photosynthetic hosts, alga-infecting *Mimiviridae* might be exposed to sunlight-induced genome damages, making these nucleases

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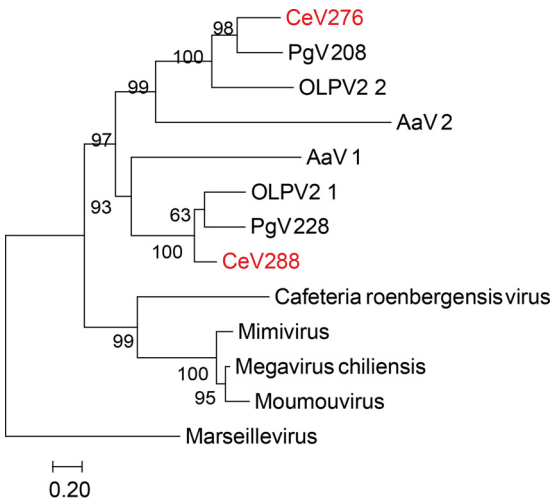

FIG 3 Duplication of the second largest subunit of the DNA-directed RNA polymerase II (RPB2) in CeV, PgV, and AaV. A maximum likelihood phylogenetic tree (58) of RPB2, aligned using Tcoffee (62) (Mcoffee mode), was constructed. Statistical branch supports (in percentages) for SH-like local support tests are given beside nodes. This phylogeny strongly supports a separate lineage leading to CeV, AaV, and PgV.

useful for repair. Such an ERCC4-dependent process extends the known diversity of viral responses to light-induced DNA damage. Other marine viruses possess a variety of mechanisms to repair DNA damage, which are either host dependent or host independent (23–25). These might be essential for the maintenance of viral communities (24), perhaps indirectly by protecting their host, as suggested by the reduced sensitivity to UV-B stress of microalgae cocultured with viruses compared to those in virus-free cultures (23).

Unrelated to the above-described function, another common feature of the alga-infecting *Mimiviridae* is the presence of two paralogs of the second-largest subunit of the DNA-directed RNA polymerase II (RPB2) (CeV_276 and CeV_288). As none of these copies appears defective and their sequences are quite divergent (only 34.2% identical), two distinct forms of transcriptional complexes might be formed following their interaction with the single RPB1 subunit encoded by these viruses. Combinations of different subunits leading to different versions of RNA polymerases IV and V (pol IV and pol V, respectively) are known in plants (26). These complexes evolved specialized roles (e.g., nonredundant gene silencing) (27, 28). Similarly, alternative RNA pol II complexes formed in alga-infecting *Mimiviridae* could play different roles. The duplicated paralogs present longer branches (Fig. 3), consistent with an accelerated rate of evolution. The presence of a C-terminal extension on the sequences of this group of paralogs would also be consistent with a functional modification: such additional residues could cause a change in substrate specificity. Phylogenetic reconstruction (Fig. 3) suggests that the RPB2 paralogs originated from a single duplication that occurred after the divergence from the rest of the *Mimiviridae*. In addition, AaV exhibits two copies of RNA polymerase II large subunits (RPB1) (AaV_242 and AaV_320) (8).

The alga-infecting *Mimiviridae* also share two distinct versions of major capsid proteins. The MCP1 paralogs exhibit a phylogeny consistent with the "species" tree and correspond to the least-divergent version of the major capsid protein common to all *Mimiviridae* (Fig. 4, top). The *Mimivirus* and *Cafeteriavirus* genera possess a second copy of MCP1, the duplication of which clearly predates their divergence (Fig. 4). In contrast, another type of MCP2 paralog (Fig. 4, bottom) is uniquely found in CeV (CeV_86, CeV_87, and CeV_88), PgV, and AaV. It probably results from an exchange with other alga-infecting large DNA viruses.

Altogether, these phyletic patterns specific to AaV, PgV, and CeV further support grouping them in a clade distinct from the other *Mimiviridae*.

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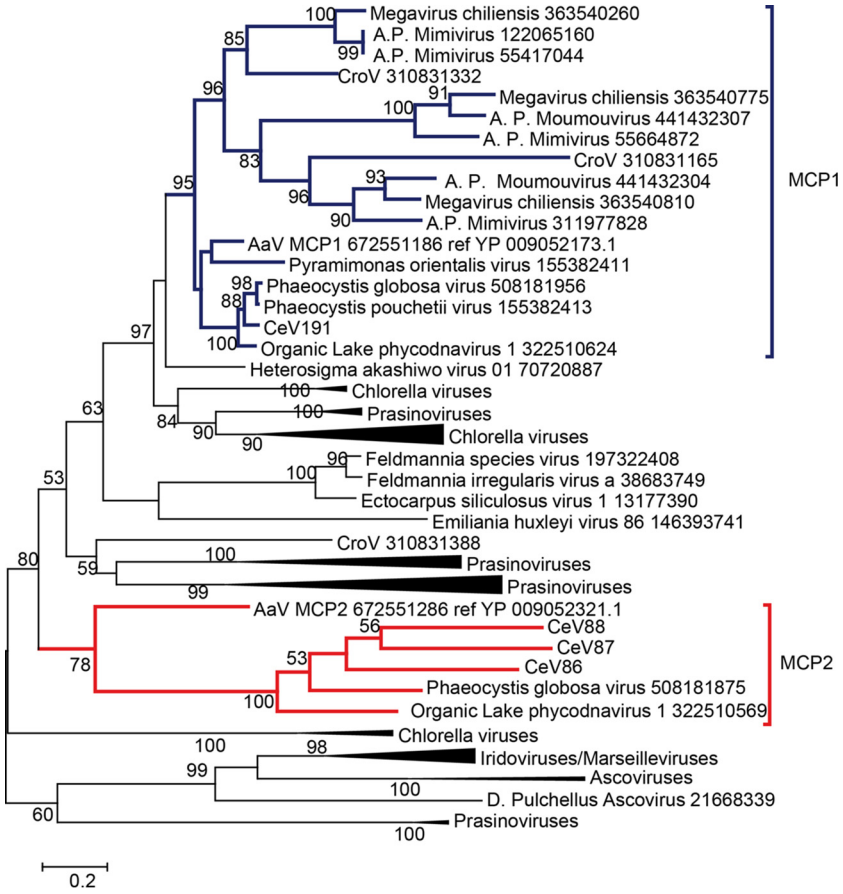

FIG 4 Relationship of the two major capsid protein homologs (MCP) found in CeV, PgV, and AaV. A maximum likelihood phylogenetic tree (58) of MCP, aligned using Tcoffee (62) (Expresso mode), was constructed. Statistical branch supports (in percentages) are given beside nodes.

Specific features shared only by CeV and PgV. CeV, PgV, and OLPV share homologs to the cold shock protein, which is known to act as an RNA chaperone (29). This protein, not found in other eukaryotic large dsDNA viruses, either was acquired by the alga-infecting lineage and later lost by AaV or was acquired after its divergence from AaV. We noticed that some dsDNA phages also encode similar cold shock proteins, suggesting that proper RNA folding is a recurrent constraint among unrelated viruses.

The analysis of PgV's genomic repeats led to the identification of an ORFan present in 12 copies, designated PgV_MIGE (major interspersed genomic element) (7). CeV possesses six copies of MIGE homologs. Interestingly, phylogenetic reconstruction grouped the PgV's MIGE and CeV's MIGE in separate clusters (Fig. 5). This suggests that MIGE was initially a single-copy gene in each virus before undergoing multiple duplications after their divergence. Analysis of CeV-MIGE did not hint at the mechanism by which this genetic element is duplicated and/or moved around. At variance with PgV, MIGE homologs in CeV lack an associated noncoding highly conserved region (7).

CeV and PgV also share a fusion event between a DNA polymerase X (DNAPolX) and a DNA ligase that is discussed in a later section.

Predicted functions unique to CeV. Among the 305 proteins unique to CeV, only 106 (35%) have a database homolog, among which only 52 are associated with functional attributes (listed in Table 2). Except for one light-harvesting complex protein (CeV_128) that will be discussed in a later section, none of these predicted functions have previously been shown to be specifically linked to the viral infection of algae.

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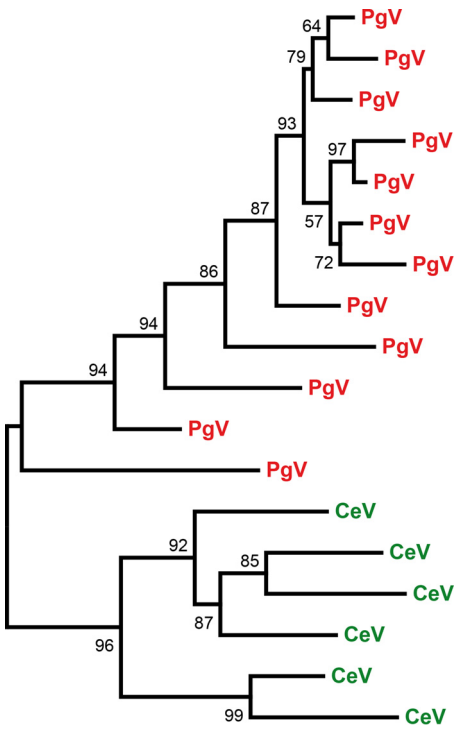

FIG 5 Independent MIGE spreading in CeV and PgV. A maximum likelihood phylogenetic tree (58) of MIGE protein sequences, aligned using Muscle (57), was constructed. Statistical branch supports (in percentages) for SH-like local support tests are given beside nodes.

CeV, a privileged host for spreading inteins? Inteins are mobile genetic elements encoding protein introns that remove themselves from host proteins through an autocatalytic excision and protein-splicing process (30). Fully functional inteins encode a homing endonuclease that mediates their integration at specific genomic sites. Different classes of inteins are found associated with the same, usually highly conserved, proteins (and thus genomic sequences), most of which are essential enzymes involved in DNA processing, replication, or synthesis. Nonallelic inteins (i.e., inteins not associated with the same insertion sites and/or protein-coding genes) do not exhibit significant sequence similarity, although they all presumably share an ancestor (30).

CeV encodes 8 inteins, to date the largest number among viruses. These inteins are inserted in 7 different ORFs: two in the ribonucleotide reductase large subunit (CeV_219), one in a DEAD-like RNA helicase (CeV_416), one in the DNA polymerase (CeV_365), one in an ATP-dependent Lon peptidase (CeV_043), one in an RPB2 paralog (CeV_288), one in a GDP-mannose 4,6-dehydratase (CeV_113), and one at the C terminus of a protein (CeV_451), concatenating an N-terminal U-box domain and a von Willebrand factor (vWA) domain. In general, intein insertions are strongly biased toward DNA-processing enzymes (30, 31). Among other explanations (30, 31), this bias might result from the fact that viruses are the main vectors of inteins within eukaryotes (32) and that viral metabolisms are mostly limited to DNA processing. In that context, the inteins hosted in a CeV's GDP-mannose 4,6-dehydratase and Lon peptidase are noticeable exceptions, to our knowledge the first such cases reported in eukaryotic viruses. With their extended metabolisms, viruses with larger genomes, such as members of the *Mimiviridae*, might thus expand the range of homing genes/enzymes for inteins. According to the current paradigm, inteins can be maintained only within essential genes. This is likely true for 5 of the 7 genes cited above (that belong to the *Mimiviridae* core genes) (3), the exception being the GDP-mannose 4,6-dehydratase that has no homolog in other *Mimiviridae*. Such a function might nevertheless be

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TABLE 2 Protein-coding genes unique to CeV and associated with functional attributes

ORF no.	Predicted function or detected domain
CeV_002	Multiple glycosyltransferase domain
CeV_003	Methyltransferase
CeV_008	Ubox domain
CeV_009	Alpha-1,2-fucosyltransferase
CeV_023	Alkylated (methylated) DNA repair protein
CeV_033	Papain-like cysteine peptidase
CeV_053	RING-finger domain
CeV_096	Putative patatin-like phospholipase
CeV_113	Intein containing GDP-mannose 4,6-dehydratase
CeV_128	Light-harvesting complex protein
CeV_137	Superoxide dismutase Cu-Zn
CeV_139	Arginase
CeV_146	Trans-2-enoyl-coenzyme A reductase (TER) and 2,4-dienoyl-coenzyme A reductase (DECR)
CeV_149	Class V aminotransferase
CeV_151	Putative phosphotransferase
CeV_152	Quaternary ammonium transporter
CeV_154	Fe-S cluster assembly scaffold protein
CeV_155	Zinc finger, C_3HC_4 type domain-containing protein, RING superfamily
CeV_161	Putative prenyltransferase
CeV_171	Phospholipase/carboxylesterase
CeV_176	Collagen and repeat-containing protein
CeV_179	Multiple type acyltransferase domains
CeV_180	Glucosyltransferase TPR
CeV_183	Glycosyltransferase family 2
CeV_184	Collagen triple helix
CeV_194	PAN/APPLE-like domain
CeV_195	Repeat containing Hsp70-like protein
CeV_196	Ubox/RING superfamily domain
CeV_201	RING-finger domain
CeV_213	Protein disulfide isomerase (PDIa)
CeV_218	Glycosyl hydrolase family 16
CeV_233	Putative AHH-like nuclease
CeV_252	Proline-rich repeats
CeV_256	Toll-like receptor
CeV_265	Partial perforin domain-like
CeV_267	Putative AAA^+ family ATPase
CeV_311	Galactose binding lectin domain
CeV_323	Putative permease
CeV_324	N-acyltransferase/N-myristoyltransferase
CeV_327	Putative AAA^+ family ATPase
CeV_359	Link (hyaluronan-binding) domain
CeV_361	Ring finger domain
CeV_366	Protein disulfide-isomerase domain
CeV_372	Hsp70-like protein
CeV_373	Acetylpolyamide aminohydrolase (histone deacetylase)
CeV_404	ATP-dependent Clp protease, proteolytic subunit
CeV_415	Putative syntaxin, SNARE domain
CeV_433	Class II HMG-box domain
CeV_440	HMG box domain
CeV_463	RING domain
CeV_464	YABBY domain
CeV_467	Catalytic core of Asn/Asp-ARNt synthetase

important, as it was independently acquired by a number of other large dsDNA viruses that infect various algae (*Chlorella* and *Prasinophyceae*) (33). The CeV homolog might thus be necessary in an alga-infecting context. The closest homologues to CeV GDP-mannose 4,6-dehydratase are intein-free bacterial enzymes (although intein-containing bacterial enzymes exist that may not have been sequenced yet).

Inteins normally insert in highly conserved regions of essential proteins. As the paradigm goes, the strong conservative constraints exerted on these regions ensures the correct excision (from the protein) or homing (into the DNA) processes. Surprisingly, we found that two similar inteins of the same prototype (standard class 1 with a

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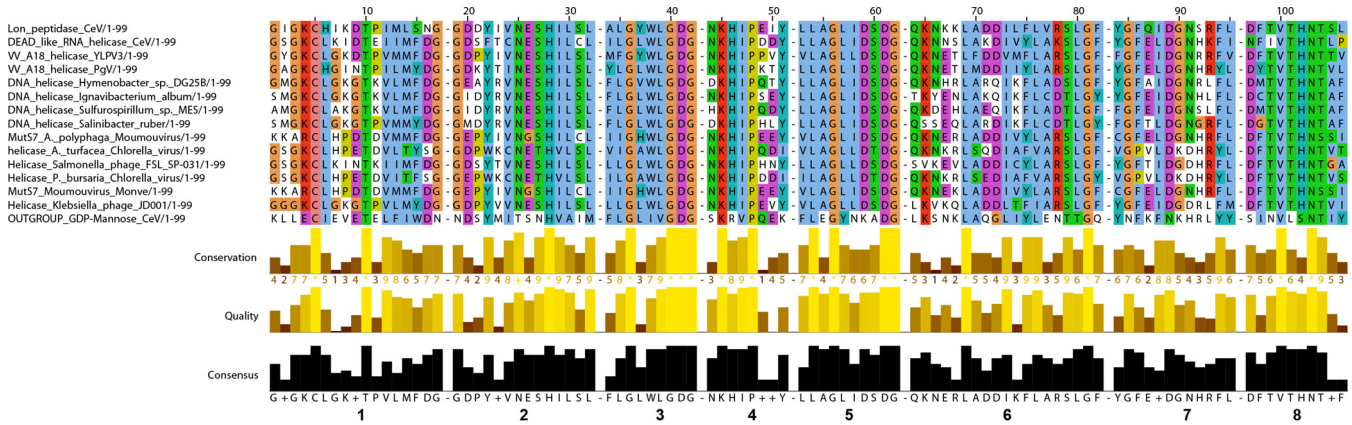

FIG 6 Helicase-type intein inserted in the CeV Lon peptidase. Multiple alignment, computed with Muscle (57), of the 8 blocks (numbered below the alignment) characteristic of inteins (Table 3). The intein hosted by the CeV GDP-mannose 4,6-dehydratase is included for comparison.

homing endonuclease of the LAGLIDADG type) have been inserted in two unrelated CeV genes/proteins: a Lon protease and a DEAD box helicase (Fig. 6 and Table 3). Moreover, a similar intein helicase-targeted allele is inserted in different enzymes in other large DNA viruses: MutS7 in Moumouvirus, the VVA18 helicase in PgV and Yellowstone Lake phycodnavirus (YLP) (percent identity with CeV Lon protease intein ranging from 42.5% to 54%), and, besides the *Mimiviridae*, DNA helicase B of 2 chloroviruses (identity with CeV Lon protease intein, 40% and 38%). Other related inteins are hosted by DNA helicases from phages or bacteria (identity with CeV Lon protease intein ranging from 39% to 43%). The 8 blocks specific to this intein prototype (34) are well conserved (Fig. 6). Although unrelated, these enzymes share a P-loop NTPase domain. All inteins but those in MutS7 are inserted in the ATP/GTP binding site (i.e., the Walker A or P-loop domain), precisely at the GK/T site. This might be sufficient for this prototype of intein to properly excise. More puzzling is the way by which this intein might have jumped from one enzyme to another. Indeed, after being cut by the intein-encoded endonuclease, the free intein gene should proceed with homing by homologous base pairing with the intein-containing allele, which serves as the template for the polymerase. This scenario appears unlikely given the limited similarity of the extein DNA sequences (Fig. 7). Another intriguing fact is that the intein is not inserted in the P-loop domain of MutS7 but is inserted at an AR/S site, 30 amino acids

TABLE 3 High similarity of the CeV Lon peptidase intein to those usually found in various DNA helicases

	% Similarity to intein no.[a]:													
Intein (no.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lon peptidase, CeV (1)	100	44	44	54	42	40	41	39	43	40	42	38	42	43
DEAD-like RNA helicase, CeV (2)	43	100	40	44	36	35	37	33	42	36	40	36	41	37
Putative VV A18 helicase, YLPV3 (3)	44	40	100	49	42	40	44	41	42	37	44	37	42	47
VV A18-like helicase, PgV (4)	54	43	50	100	43	44	46	41	42	42	46	42	42	45
DNA helicase, *Hymenobacter* sp. (5)	42	35	42	43	100	59	62	58	36	33	41	33	35	43
DNA helicase, *Ignavibacterium album* (6)	40	36	40	43	59	100	60	62	37	32	41	32	36	45
DNA helicase, *Sulfurospirillum* sp. (7)	41	36	44	46	62	60	100	55	34	32	38	32	34	45
DNA helicase, *Salinibacter ruber* (8)	39	33	41	41	58	62	55	100	34	33	37	33	34	42
MutS7, A. P. Moumouvirus (9)	43	42	42	42	36	36	34	34	100	37	37	36	94	40
Helicase, ATCV NTS-1 (10)	40	35	37	42	33	31	32	33	37	100	35	75	37	51
Helicase, *Salmonella* phage (11)	42	40	44	46	41	40	38	37	37	35	100	36	38	36
Hypothetical protein B508R, PBCV (12)	38	35	37	42	33	32	32	33	36	75	36	100	35	41
MutS7, Moumouvirus Monve (13)	42	41	42	42	35	36	34	34	94	37	38	35	100	25
Helicase, *Klebsiella* phage (14)	43	37	47	45	43	44	45	42	40	37	51	36	41	100
Outgroup GDP-mannose CeV					24	26	22	20				25		20

[a]The pairwise percentages of identity between these homologous inteins is given. The values for the nonallelic intein hosted by CeV GDP-mannose 4,6 dehydratase is included for comparison when pairwise alignment was possible. YLPV3, Yellowstone Lake phycodnavirus 3; ATCV, Acanthocystis turfacea chlorella virus; PBCV, Paramecium bursaria chlorella virus.

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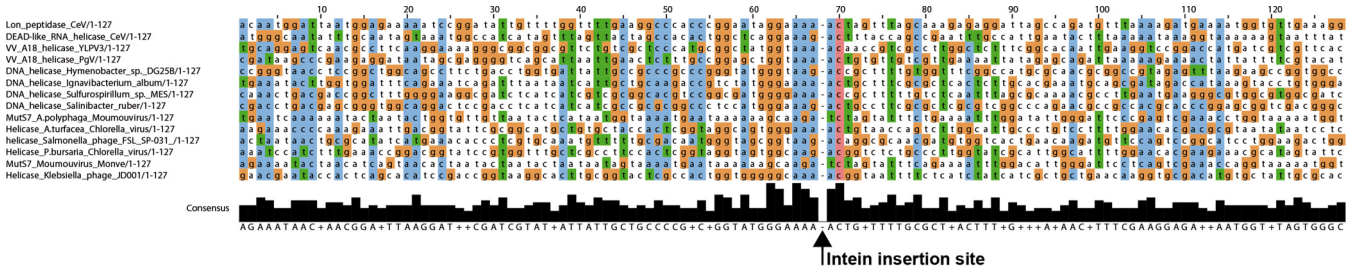

FIG 7 Extein DNA sequences in different genes hosting similar intein alleles (Fig. 6) are not similar.

upstream. Furthermore, these amino acids are not conserved in the intein-free MutS7 found in other *Mimiviridae*. Altogether, these cases constitute violations of the current allele-specific intein paradigm. They suggest a mechanism specific to the *Mimiviridae* spreading inteins while widening their homing range.

Convergent acquisition of host genes. Detailed attention was paid to the CeV-encoded light-harvesting complex protein (LHC) (CeV_128) of the LIL (light-harvesting light) family. The presence of such a gene coding for a component of the photosynthesis apparatus was rather unexpected. Further analyses led to the discovery that other alga-infecting viruses encode proteins of the LIL family (Fig. 8). Their phylogenies clearly suggest that they were acquired from their hosts through three independent events (one for CeV and two for the prasinoviruses) (Fig. 8). Besides the expected chlorophyll binding (CB) motifs and 3 predicted transmembrane helices (35), all viral LIL proteins contain an N-terminal transit peptide targeting them to their respective host chloroplast type (36) (secondary red for *H. ericina* and primary green for the prasinophytes). This suggests that these proteins are functional. Rather than collecting incom-

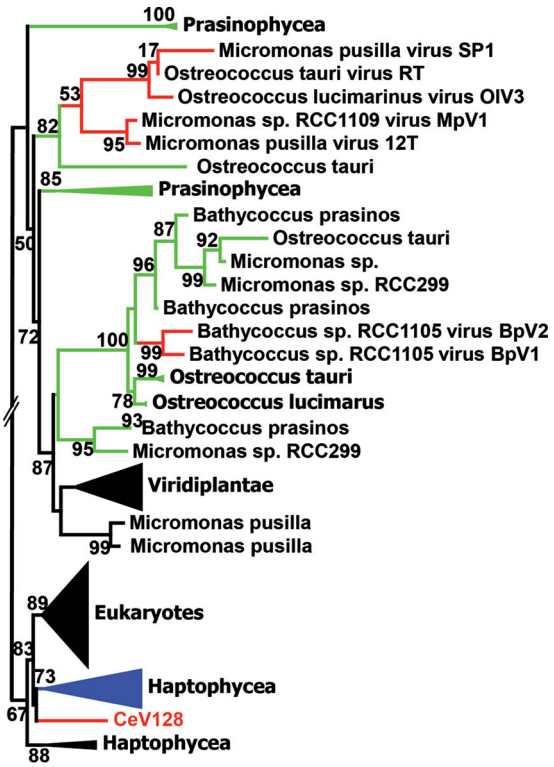

FIG 8 Convergent acquisitions of host LIL proteins. A maximum likelihood phylogenetic tree (58) of LIL proteins, aligned using Muscle (57), was constructed. Statistical branch supports (in percentages) for the S-H-like local support tests are given beside nodes. Branches corresponding to viruses are colored red, with blue for haptophyceae and green for prasinophyceae. Sequences used for the analysis were selected using BLAST Explorer as implemented in Phylogeny.fr (63).

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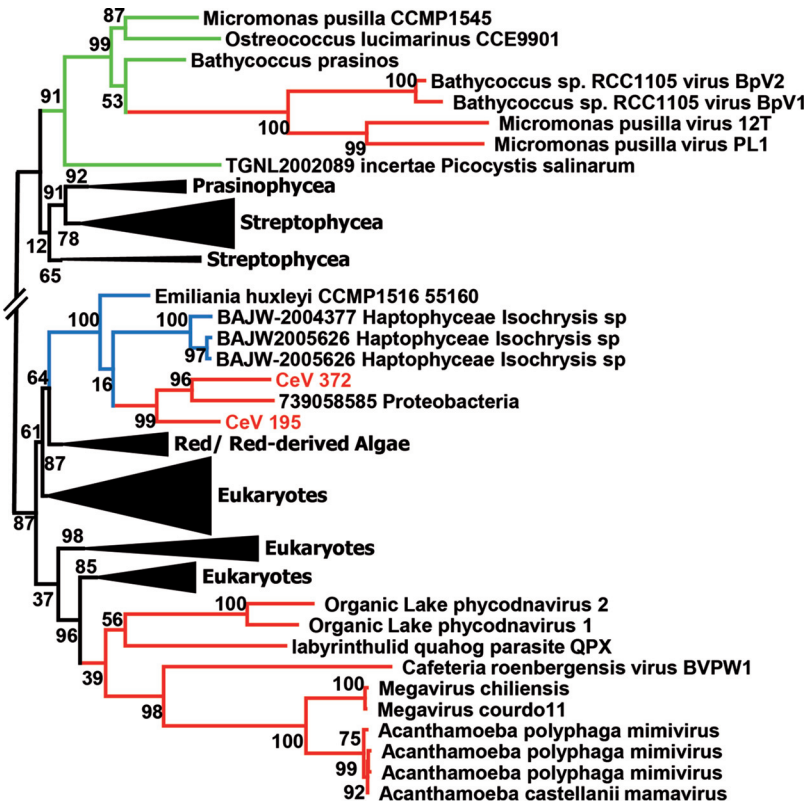

FIG 9 Convergent acquisitions of host HSP70 proteins. A maximum likelihood phylogenetic tree (58) of HSP70, aligned using Muscle (57), was constructed. Statistical branch supports (in percentages) for S-H-like local support test are given beside nodes. Viral branches are colored red, with blue for haptophyceae and green for prasinophyceae.

ing light for photosynthesis, some nucleus-encoded eukaryotic LIL proteins are involved in photoprotection, such as nonphotochemical quenching (NPQ) (35, 37). This might be the role of the viral LIL homologs.

We then systematically looked for additional cases of convergent acquisition involving other genes. We identified 2 more, concerning the DNAK (HSP70) chaperone (CeV_372 and CeV_195) (Fig. 9) and a U-box domain at the C terminus of the CeV_008-encoded protein (data not shown). CeV and two prasinoviruses have independently acquired HSP70, likely from their hosts (Fig. 9). Interestingly, other *Mimiviridae* also encode homologs of this chaperone. However, these proteins appear to derive from an ancestral version of HSP70 already present at the root of the family tree. Thus, the HSP70 proteins found today in CeV might result from a nonhomologous replacement of the ancestral *Mimiviridae* version of the protein. The U-box domain mediates the ubiquitin conjugation of protein targeted for degradation in the proteasome (38). CeV (CeV_008) and EhV (EMVG_00184) appear to have independently acquired host-derived U-box domains.

Gene fusions in CeV. Gene fusions are genomic rearrangements that are thought to facilitate the coexpression and/or assembly of proteins initially encoded separately. The involved proteins could physically interact or be functionally associated (39, 40).

We systematically screened for potential gene fusions in the CeV genome by comparing the topology of homologous genes in other *Mimiviridae*. Two clear cases were identified: one (CeV_489) is a fusion between the DNAPolX and the NAD-dependent DNA ligase (also found in PgV), and the other (CeV_007) is a fusion between the uridyltransferase and the UDP-glucose 4,6-dehydratase (UDG). We did not detect such fusions in other viruses or cellular organisms.

The DNAPolX/NAD-dependent DNA ligase fusion makes functional sense, as these enzymes normally work in succession when participating in DNA repair: DNAPolX

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fills single-nucleotide gaps before their ends are sealed by the ligase (41, 42). CroV homologs of these two proteins are encapsidated, suggesting their role in prereplicative DNA repair (43). We noticed that the CeV and PgV fusion proteins exhibit very different linkers that could have resulted from 2 independent events. However, the analysis of similar fusions in the environmental database showed that such linkers are not conserved. The CeV and PgV proteins thus likely resulted from a unique fusion event prior to their divergence and that of their linkers. Interestingly, some bacteria also exhibit a fusion between an ATP-dependent DNA ligase and a DNA polymerase domain (44). This might constitute a case of functional evolutionary convergence between bacteria and eukaryotic viruses resulting in more efficient DNA repair enzymes.

The unique fusion between a uridyltransferase domain and UDG identified in CeV (CeV_007) might optimize the synthesis of L-rhamnose, known to be involved in the glycosylation of structural proteins in mimivirus and certain chloroviruses (45). This fusion occurred after the duplication of the uridyltransferase gene (CeV_479), originally involved in the three-component UDP-*N*-acetylglucosamine biosynthetic pathway (46) found in large *Mimiviridae*. This duplication might have opened the way to the fusion event (47).

DISCUSSION

Convergent evolution in CeV and other large dsDNA viruses. This study identified several cases where homologs of the same gene were independently acquired by CeV and other viruses, most likely from their hosts. The best example of such convergent acquisition is that of the light-harvesting complex protein (LHC) that is found in CeV and seven species of prasinoviruses (Fig. 8). Such gene transfers are reminiscent of the acquisition of core photosystem components (including a remote member of the LIL family, HLIP) by cyanophages from their bacterial hosts (35, 37, 48). Thus, the manipulation of host photosynthesis by viruses might be a recurrent theme in evolution, most likely to meet the increased energy burden of the production of virions by the infected cell.

DNA repair is another general function that has been the object of convergent innovations, this time through gene fusions. The DNAPolX-NAD-dependent DNA ligase fusion identified in CeV (and shared with PgV) is echoed by that of a DNAPolX-AP-endonuclease found in poxviruses (49) and that of an Mre11 and Rad50 domain in mimivirus (50). These proteins are likely parts of an optimized dsDNA strand break repair machinery.

The unique fusion between a uridyltransferase domain and UDG identified in CeV (CeV_479), the presence of several enzymes for the synthesis of rhamnose in mimivirus (45), and the fused sequential enzymes found in OtV5, OmV1, and OlV1 (YP_001648294.1, YP_009172960.1, YP_004061822.1) are other examples of convergent innovations targeting the same biosynthetic pathway, probably central to the glycosylation of viral structural proteins (45).

CeV also acquired copies of the HSP70 chaperone (CeV_195 and CeV_372) that clearly originated from a different source than the one encoded by its close relative, OLPV, as well as CroV and acanthamoeba-infecting *Mimiviridae*. It is also different from the one acquired by several prasinoviruses from their hosts (Fig. 9).

Finally, CeV and EhV have independently acquired U-box domain-encoding sequences, suggesting the active involvement of the host proteasome in the infectious process, perhaps as a source of recycled amino acids (51).

The puzzling origin of CeV's many unique genes. We previously pointed out the paradox of CeV exhibiting so many unique protein-coding genes (294/512, or 57.4%), while the rest of them overwhelmingly had their closest relative in PgV or other *Mimiviridae*. This paradox is amplified by the small number of core genes, i.e., genes common to all of these obviously related viruses (Fig. 2). This paradox would be partially explained if most of these so-called unique genes were not real but were false-positive calls from an overestimating (albeit standard) (9) bioinformatic annotation procedure. Such ambiguities are best solved by validating gene predictions using

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transcriptomic data that unfortunately is not available for CeV. However, we can estimate the actual proportion of real genes among the unique ones using CroV, for which such data exist. According to the original publication (5), CroV has 544 protein-coding genes, of which 438 genes were tested for transcription and 274 (63%) found positive. We then examined how this proportion changed when separately considering unique versus shared CroV genes. Out of the 438 tested genes, 299 are unique, of which 177 (59.2%) fall in the expressed category. On the other hand, 97 of the 139 shared genes were found to be expressed (69.7%). Even though these numbers denote a significant difference in transcript detection in favor of shared genes (P value of <0.05 for the table [177, 122; 97, 42] by Fisher's exact test), they nevertheless suggest that a large proportion (59.2/69.7, or 84.9%) of CroV unique genes are real (postulating that their transcripts should be detected in the same proportion as those of shared genes). Extending the same reasoning to CeV and using the same ratio would reduce the number of unique true genes from 294 to 250, i.e., still half of its total gene complement.

The evolutionary scenario susceptible to leading to the presence of so many CeV-unique genes while preserving the strong phylogenetic affinity globally exhibited by the genes shared among the *Mimiviridae* remains to be elucidated. As most of the unique CeV genes are ORFans, a scenario involving a huge number of gene acquisitions from cellular organisms (or known viruses) is unlikely, short of postulating an equally huge mutation rate erasing their phylogenetic origins. There is no evidence of such genomic instability in these large dsDNA viruses. On the contrary, they are well equipped with high-fidelity DNA replication and repair machineries (our results here and reference 52). The converse model, postulating a reductive evolution from a common *Mimiviridae* ancestor (2) with a genome large enough to accommodate the number of unique genes indicated in Fig. 2, appears increasingly unlikely (although viruses with thousands of genes are known to exist [53]). An alternative to the opposite and equally unlikely accretion and reduction evolutionary scenarios would be to postulate that these DNA viruses *de novo* generate new protein-coding genes (and functions) by a totally unknown mechanism. Demonstrating such a capacity would definitely put viruses on the center stage of biological evolution.

Proposed taxonomy of an extended *Mimiviridae* family. The present analysis of the CeV genome adds to the mounting consensus that despite their large differences in gene content, particle size, host range, and ecology, a group of alga-infecting large dsDNA viruses (CeV, PgV, OLPV, and AaV) and the acanthamoeba-infecting *Mimiviridae* (genus, *Mimivirus*) belong to the same family and share an ancestor (3, 7, 8, 13). This family also includes CroV (5), a virus that infects the heterotrophic stramenopile *Cafeteria roenbergensis*, as the sole member of the genus *Cafeteriavirus*. In addition to the sharing of unique genes (such as the mismatch DNA repair protein MutS7 and the puzzling asparagine synthase), large A+T rich genomes, and a full DNA transcription and replication apparatus, divergent members of this virus group (mimivirus, CroV, Pgv, and OLPV) exhibit a unique association with virophages, small dsDNA viruses replicating as parasites of their intracytoplasmic virion factories. Since CeV, PgV, and their relatives (such as Phaeocystis pouchettii virus or the Organic Lake phycodnaviruses) infect unicellular algae, they are referred to as unclassified new members of the family *Phycodnaviridae* in the literature as well as in sequence databases. As presently recognized by the ICTV, the family *Phycodnaviridae* includes six genera: *Raphidovirus*, *Coccolithovirus*, *Phaeovirus*, *Chlorovirus*, *Prasinovirus*, and *Prymnesiovirus*. As more alga-infecting viruses are characterized, it is clear that an increasing number of them do not fit within this established family, the name of which ("phyco" means "algae") has become a source of confusion. This highlights the danger in classifying viruses within clades named after their hosts, as there is increasing evidence that the same host can be infected by phylogenetically distinct viruses (such as *Acanthamoeba* being infected by five different types of giant dsDNA viruses: mimivirus, marseillevirus, pandoravirus, pithovirus, and mollivirus) (2). Conversely, the *Mimiviridae* family (described here) (but also the *Asfarviridae* family [54]) shows that viruses with

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strong phylogenetic relationships can infect hosts belonging to branches that diverged at the earliest time of eukaryote history.

To help clarify the classification of alga-infecting viruses and acknowledge the phylogenetic affinity of CeV, PgV, and AaV with CroV and the mimivirus group (Fig. 1), we propose to divide the family *Mimiviridae* into two subfamilies. One, tentatively named the "Megamimivirinae" (i.e., the largest *Mimiviridae*), should include the 3 clades (A, B, and C) of the existing *Mimivirus* genus (4) and CroV as the prototype of the existing *Cafeteriavirus* genus. A new subfamily, named the "Mesomimivirinae" (i.e., the still large but smaller *Mimiviridae*), should include CeV and PgV (as well as the partially sequenced OLPV1 and OLPV2) as a new genus and the outlier AaV as the prototype of yet another distinct genus. Redefined as proposed, the new family *Mimiviridae* would clearly separate the above-described large alga-infecting viruses from the *Phycodnaviridae* family while acknowledging their relationship with the acanthamoeba-infecting mimiviruses. At the same time, the range of sequence divergence exhibited by the core proteins (such as DNA pol B, MutS7, or the packaging ATPase) within the *Mimiviridae* will remain comparable to that observed within other large dsDNA virus families, such as the *Poxviridae* (also divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*). As this paper was in review, findings from metagenomic studies suggested that yet another lineage of large *Mimiviridae* remains to be characterized (55).

MATERIALS AND METHODS

Genome sequencing, assembly, and annotation. The procedures for genome sequencing, assembly, and annotation were described previously in reference 9.

Identification of gene fusions. We mapped the CeV predicted proteins onto other *Mimiviridae* genomes using TBLASTN. When different segments of a CeV protein were found to best match at two distant locations in the target genome, the corresponding ORFs were submitted to further phylogenetic analyses to confirm their orthologous relationship with the different parts of the candidate CeV fusion protein. BLAST best-scoring sequences belonging to bacteria, archaea, eukaryotes, and viruses were included in the analysis to establish that the fusion occurred within the *Mimiviridae* lineage.

S-H test. To discriminate between the three possibilities, (i) OLPV emerged first after AaV, (ii) PgV emerged first, or (iii) CeV emerged first, we performed multiple Shimodaira and Hasegawa (S-H) tests (56) as follows. First, we identified the 39 clusters of orthologs present in the proteomes of AaV, OLPV, PgV, and CeV using OrthoMCL. Second, 4 proteins of each of the 39 clusters were aligned using MUSCLE (57), the resulting 39 multiple alignments were visually validated, their gapped positions removed, and the corresponding likelihood matrices for the three tree topology were computed with PhyML (58).

Third, the CONSEL procedure (59) (www.sigmath.es.osaka-u.ac.jp/shimo-lab/prog/consel/) was applied to perform the S-H test itself (i.e., a computation of the *P* value for each of the possible topologies by comparison of the matrix of the log likelihoods).

The test was not conclusive. Among the 117 (3×39) trees, we could reject only three of them at a *P* value of <0.05.

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