

Isolation of Prasinoviruses of the Green Unicellular Algae *Ostreococcus* spp. on a Worldwide Geographical Scale^{∇†}

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***Ostreococcus* spp. are extremely small unicellular eukaryotic green algae found worldwide in marine environments, and they are susceptible to attacks by a diverse group of large DNA viruses. Several biologically distinct species of *Ostreococcus* are known and differ in the ecological niches that they occupy: while *O. tauri* (representing clade C strains) is found in marine lagoons and coastal seas, strains belonging to clade A, exemplified by *O. lucimarinus*, are present in different oceans. We used laboratory cultures of clonal isolates of these two species to assay for the presence of viruses in seawater samples from diverse locations. In keeping with the distributions of their host strains, we found a decline in the abundance of *O. tauri* viruses from a lagoon in southwest France relative to the Mediterranean Sea, whereas in the ocean, no *O. tauri* viruses were detected. In contrast, viruses infecting *O. lucimarinus* were detected from distantly separated oceans. DNA sequencing, phylogenetic analyses using a conserved viral marker gene, and a Mantel test revealed no relationship between geographic and phylogenetic distances in viruses infecting *O. lucimarinus*.**

Viruses are the most abundant and genetically diverse biological entities in marine environments (48). The three ways most often used to assess eukaryotic algal virus diversity are (i) using a functional host-virus system to quantify viruses specific to one host strain (i.e., culture-based studies) (4), (ii) using PCR amplification and sequencing a conserved gene (10, 12–14, 28), and (iii) using whole-community genome sequencing (i.e., metagenomics) (6, 8, 31). Recently, the advent of sequencing techniques like shotgun sequencing or pyrosequencing (38) has led to an increase in the number of metagenomics projects. The Global Ocean Sampling (GOS) Expedition has provided a unique opportunity to investigate viral diversity in different environments within the size fraction of 0.1 to 0.8 μm (39). The GOS data revealed highly abundant viral sequences (at least 3% of the predicted proteins had a viral origin) (53). In another study, the analysis of marine viromes from four oceanic regions suggested that the composition of viral assemblages depends on their geographic locations, but these authors conclude that similar viruses are widespread throughout the oceans (2). Despite these new methods and different ways to analyze viral diversity, we still do not really know if “everything is really everywhere” (7).

The present study addresses a specific part of this problem: are viruses infecting a single host strain present at geographically distant locations? If several viral strains are identified and characterized, how closely do these viruses resemble one another on a phylogenetic scale? In order to answer these questions, we focused on a microalgal (*Prasinophyceae*)-virus (*Prasinovirus*) system. The studied hosts belong to the genus *Ostreococcus*, a ubiquitous prasinophyte picoeukaryotic alga

abundant throughout the oceanic euphotic zone (55, 56). Several strains from this genus were isolated and assigned to four distinct ecotypes according to their growth parameters under different light regimens (22, 36), which correspond to four well-defined phylogenetic clades in an internal transcribed spacer (ITS)-based phylogeny (clades A to D). The complete genome sequences of two *Ostreococcus* species have been described: *O. tauri* (19) and *O. lucimarinus* (35). In the present study, viruses infecting specific host species (*Ostreococcus* spp.) have been screened from a variety of locations around the world.

Among viruses infecting *Ostreococcus*, the genome of a single strain (OtV5) has been fully sequenced (18), and the phylogenetic relationships among several virus strains have been investigated (4). These viruses belong to the *Prasinovirus* group, a genus of the *Phycodnaviridae* family. Many viruses infecting phytoplankton are members of the *Phycodnaviridae*; they have double-stranded DNA genomes and large polyhedral capsids (20). In the prasinophyte-*Prasinovirus* system, the hosts and viruses can be grown on solid medium and are easily maintained in the laboratory. *Ostreococcus* viral strains have been isolated and characterized by phylogenetic analysis based on their B-family DNA polymerase (*DNA pol*) partial gene sequence (4). This DNA polymerase is a useful marker for phylogenetic analyses because its sequence is well conserved in all known members of nucleocytoplasmic large DNA viruses (NCLDVs) (26), including *Phycodnaviridae*. Furthermore, several previous studies have examined the abundance and the genetic diversity of marine eukaryotic viruses using environmental sequencing approaches and amplified *DNA pol* gene fragments (11, 12, 43–46), and Monier et al. (31) used this marker to describe the taxonomic distribution of large DNA viruses from the GOS data.

The first stage of this study was to isolate *Ostreococcus* viruses from different worldwide geographic locations, by culturing on various host strains. In a second stage, these viral strains were characterized via the sequencing of their *pol* sequence (encoding a part of their DNA polymerase gene), and their

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specificity toward different host strains was assessed in order to assess the potential host range of the viral strains isolated and to gain a better understanding of their population dynamics and distribution. Finally we compared these new *Prasinovirus* DNA sequences with metagenomic sequence data (obtained from sampling all around the world) and environmental sequence data identified using BLAST similarity to assess the global distribution of similar *Ostreococcus* viruses.

MATERIALS AND METHODS

Study sites. Eight seawater samples were collected between September 2008 and February 2009 from different near surface depths from 3 sites within the Pacific, 3 from Atlantic, 1 from English Channel, and 1 from Mediterranean Sea (see Table S1 in the supplemental material). All samples were collected using Niskin bottles and were kept in the dark at room temperature until subsequent analysis was performed within 1 to 7 days after collection.

Culture of the algal host and production of viral lysis plaques. *Ostreococcus tauri* strain RCC745 (Roscoff Culture Collection [51]) and *O. lucimarinus* CCMP2972 (Center for Culture of Marine Phytoplankton) were used as hosts for the infection experiments. Cultures were maintained in Keller's medium (27) prepared in autoclaved and 0.22- μm -pore-filtered seawater under a permanent irradiance of 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Luminex cool white fluorescent tubes, type L36W/840). Routine analysis for plaques was done using a protocol modified from Derelle et al. (18). Seawater samples were prefiltered by gravity through membranes with a porosity of 3 μm (Millipore type SSWP) and then filtered again through a 0.45- μm filter (Millipore type SSWP). Ten milliliters of this filtrate was mixed with 7 ml of Keller's medium and 8 ml of 3.10^7 cells/ml of a growing host culture. A 1.5% solution of agarose (Euromedex type D-5, DNA grade) was dissolved in distilled water by heating to 100°C and held at 60°C in a water bath. Rapidly, 3 ml of the warm agarose solution was added to the seawater-medium mixture and poured immediately in a 9-cm-diameter petri dish. Collection and filtration of the water and plating out for visualization of lysis plaques were performed within 2 h. A minimum of 4 plates were prepared from each seawater sample and from each host tested. The plates were cultured (continuous light at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ at $20 \pm 1^\circ\text{C}$) inside a transparent plastic box to maintain humidity for 1 to 2 weeks. A few days after plating, plaques often appeared inside the agarose gel. These were picked off, added to 400 μl of a solution of MgSO_4 (SM buffer; CSH protocols, 2006; doi:10.1101/pdb.rec466) and conserved at 4°C. In order to obtain viral lysate for subsequent experiment on specificity, 200 μl of this lysis plaque was added to a host culture (in the exponential phase) of 6 ml, and few days later, this yielded 6 ml of cleared viral lysate.

Identification of viral sequences in environmental sequence databases. First, we screened a metagenomics database (<http://camera.calit2.net/>) for potential DNA *pol* sequences from *Ostreococcus* viruses. A conservative approach was taken in order to distinguish only *Ostreococcus* viral sequences. A sequence was considered for further analysis only if its top BLASTN hit was clearly attributed to a viral DNA polymerase with a minimum size of 500 bp and an E value of $<1\text{e}-20$. Second, DNA *pol* viral environmental sequences amplified using the same primer set in this study were downloaded from GenBank. Many sequences from an "unknown host" were found, originating from several independent sampling series (12, 17, 45, 46). All of these sequences (from GenBank and metagenomics data) were used to generate an initial phylogenetic reconstruction, and then only one representative sequence from the clade closely related to *Ostreococcus* viruses was kept to build the final phylogenetic reconstruction.

PCR amplification. Group-specific primers (AVS) already designed on the basis of viruses infecting *Micromonas pusilla* (MpV-Sp1), two *Chlorovirus* strains (PBCV-1 and NY-2A) (10, 11), and viruses infecting *Ostreococcus* (4) were used to amplify viral DNA polymerase fragments from lysis plaques. These primers AVS1 (5'-GARGGIGCIACIGTIYTGAYGC-3') and AVS2 (5'-GCIGCRTAI CKYTTYTTISWRTA-3') amplify a DNA polymerase fragment about 600 bp long. PCR conditions were described within a previous study (4). PCR bands were purified directly by using the NucleoSpin kit (Macherey-Nagel Company, Düren, Germany), and DNA fragments were sequenced (Macrogen, Inc., South Korea) using the amplification primers. To control for PCR or sequencing errors, fragments were sequenced in the reverse and forward senses, and all nucleotide differences were checked visually using the chromatograms. Special care was taken to avoid contamination or mislabeling: all experiments (from picking of viral lysis plaques to DNA amplification and purification) were independently run at least twice, by two different persons. The results were always similar.

Phylogenetic reconstruction. Sequences were read and corrected using BioEdit (version 7.0.0) (25). BLASTN (NCBI, <http://www.ncbi.nlm.nih.gov/>) was used to search for similar sequences in public databases (GenBank and CAMERA) (see Table S2 in the supplemental material). Amino acid sequences were then manually aligned using Se-Al (A. Rambaut, 1996; Se-Al: Sequence Alignment Editor [<http://evolve.zoo.ox.ac.uk/>]). Phylogenetic reconstructions based on amino acid sequences were carried out by Bayesian inference and maximum likelihood. Bayesian analysis was done with MrBayes 3.1.2 (37), with 4 chains of 2.10^6 generations and trees sampled every 200 generations, with the burn-in value set to 20% of the sampled trees. Using Bayesian inference, protein sequences were analyzed with a mixed-amino-acid model (37), and DNA sequences were considered with an evolutionary model designed for coding sequences taking the genetic code into account (21, 32, 42). Maximum likelihood reconstructions were carried out only on amino acid sequences, using PhyML (23, 24) with an evolutionary model selected via Akaike Information Criterion with ProtTest (1), and validated with 100 bootstrap replicates.

Statistical analysis. To look for a biogeographic pattern, we used a Mantel test (30) to study the correlation between geographical distances and patristic distances computed from the phylogenetic tree, using TreeEdit (Rambaut and Charleston, 2001; <http://tree.bio.ed.ac.uk/software/treededit/>). The Mantel test was computed using the function "mantel" from the "vegan" library (J. Oksanen, R. Kindt, P. Legendre, and R. B. O'Hara, 2007; vegan: Community Ecology Package [<http://cran.r-project.org/>]) of the R statistical language v2.8.1 (R: a language and environment for statistical computing, 2008; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [<http://www.R-project.org/>]). Significance was assessed using 1,000 permutations.

Specificity. Virus susceptibilities were examined by using a plating technique on a range of prasinophytes. Plates with host cells were prepared (7 ml of Keller's medium, 8 ml of a 3.10^7 cells/ml of a growing host culture, and a 1.5% solution of agarose [Euromedex type D-5; DNA grade]) to which we added 2 μl of virus in duplicate. The plates were cultured (continuous light 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, at $20 \pm 1^\circ\text{C}$) inside a transparent plastic box to maintain humidity for 10 days. Plates that were not lysed 10 days after viral inoculation were considered to be resistant to the virus.

Nucleotide sequence accession numbers. All sequences used in this study were deposited in GenBank. The accession numbers of virus DNA polymerase partial gene sequences are as follows: GQ412082, OIV349; GQ412083, OIV350; GQ412084, OIV359; GQ412085, OIV360; GQ412086, OIV364; GQ412087, OIV368; GQ412088, OIV402; GQ412089, OIV468; GQ412090, OIV470; GQ412091, OIV462; GQ412092, OIV464; GQ412093, OIV465; GQ412094, OIV466; GQ412095, OIV467; GQ412096, OIV536; GQ412097, OIV537; GQ412098, OIV458; GQ412099, OIV158; GQ412100, OIV164; and GQ412101, OIV155.

RESULTS

Isolation and characterization of prasinoviruses. Water samples obtained from eight locations from different oceans (South and North Pacific, South and North Atlantic, Mediterranean Sea, and English Channel) were screened for the presence of viruses infecting two prasinophyte species, *Ostreococcus tauri* and *O. lucimarinus*, isolated from the Thau lagoon (Mediterranean Sea) and the North Pacific, respectively (see Table S1 in the supplemental material). Viruses of *O. tauri* were found only in samples from the Mediterranean Sea station, but viruses of *O. lucimarinus* were isolated from four locations (South Pacific, North Atlantic, English Channel, and Mediterranean Sea). We thus focused this study on *O. lucimarinus* viruses. Twenty viruses were isolated, and a fragment of the viral DNA *pol* gene was amplified and sequenced. The phylogeny of these 20 viral sequences (6 from the North Atlantic, 4 from the South Pacific, 5 from the English Channel, and 5 from the Mediterranean Sea) (Fig. 1) did not form clades that were associated with their geographic origins, except for North Atlantic sequences, which clustered together. We tested the correlation between geographical distances and phylogenetic distances via a Mantel test, which confirmed the absence of such a link ($P = 0.338$) and therefore supports the

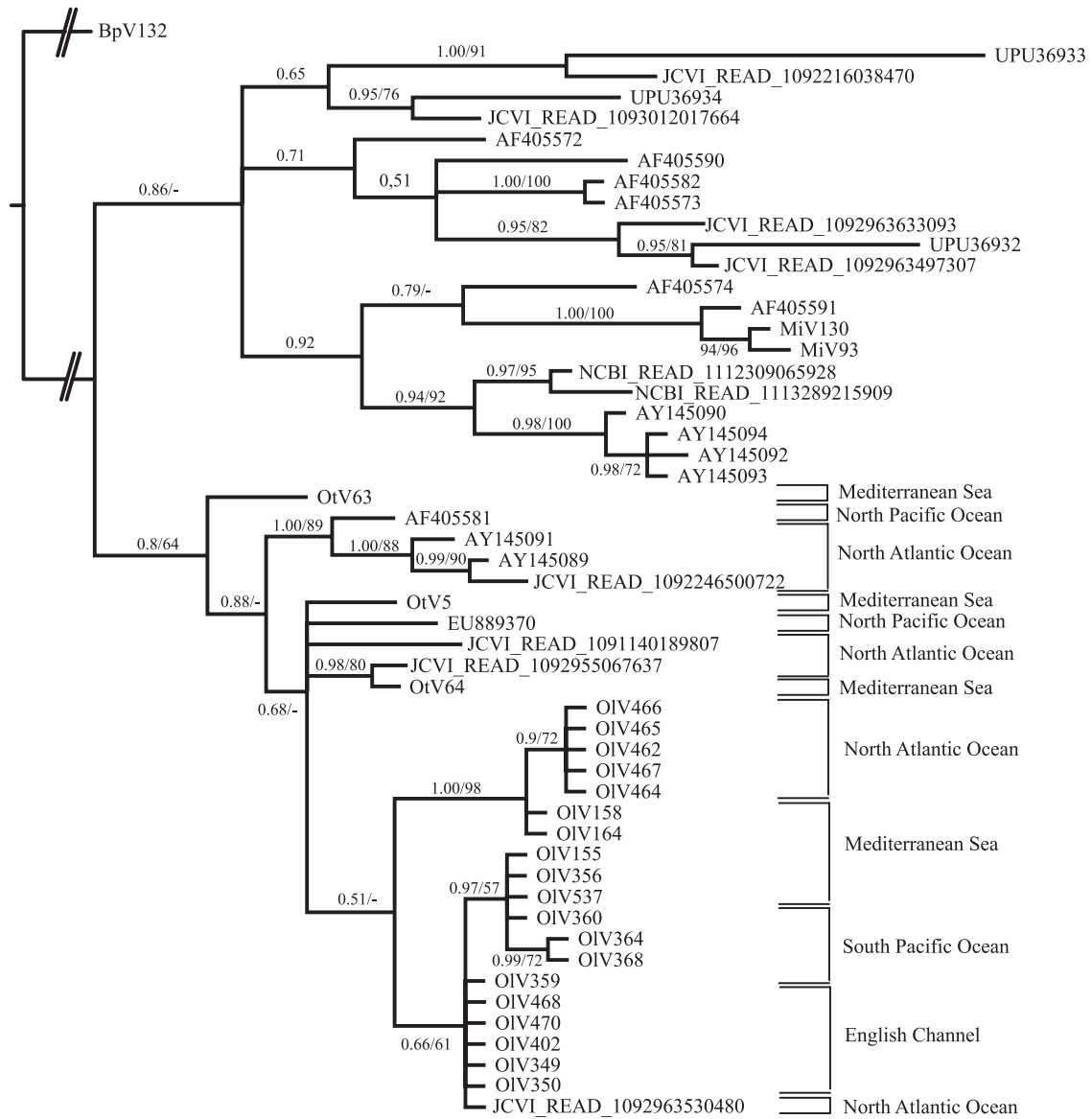


FIG. 1. Phylogenetic tree of prasinoviruses with environmental sequences from the partial DNA polymerase gene (for a final length of 195 amino acids [aa]) reconstructed by Bayesian inference (BI) and maximum likelihood (ML). Numbers are posterior probabilities (BI) and bootstrap proportions (ML) reflecting clade support (ML; values below 50 are indicated by dashes). Geographic origins of sequences are given on the right. GenBank accession numbers are as follows: MiV130, FJ267495; MiV93, FJ267493; OtV63, FJ267501; OtV64, FJ267502; BpV132, FJ267517; and OtV5, EU304328.

absence of a biogeographical pattern. Viral sequences from the Mediterranean Sea displayed more variability (mean nucleotide divergence of 10.84%), than those from the North Atlantic (0.18%) and South Pacific (3.54%) (Table 1). Sequences from the English Channel were almost all identical, with a mean divergence of 0.07% and a maximum divergence of 0.18%. The mean nucleotide divergence between sequences from South Pacific and English Channel was only 4.12%, but between North Atlantic and English Channel sequences, the mean nucleotide divergence was 20.12%. The nucleotide divergence of 10.84% reported above for 5 viruses from *O. lucimarinus* at the Mediterranean Sea site is higher than what was found in a previous study (L. Bellec, N. Grimsley, E.

TABLE 1. Nucleotide divergence among partial DNA polymerase gene sequences of viruses from different locations

Virus source location(s)	% Nucleotide divergence		
	Maximum	Mean	SD
English Channel	0.18	0.07	0.09
South Pacific	4.86	3.54	1.86
North Atlantic	0.51	0.18	0.26
Mediterranean Sea	18.01	10.84	9.03
English Channel and South Pacific	5.04	4.12	1.35
English Channel and North Atlantic	20.36	20.12	2.1
English Channel and Mediterranean Sea	18.37	10.91	6.2
South Pacific and North Atlantic	20.72	20.49	2.56
South Pacific and Mediterranean Sea	18.91	9.57	7.7
North Atlantic and Mediterranean Sea	19.45	13.53	7.72

Derelle, H. Moreau, and Y. Desdevises, submitted for publication) at the same sample site, where 65 viruses of *O. tauri* were isolated, displaying a nucleotide divergence of only 1.61%.

Host origin and specificity. Eight prasinophyte host strains representing *Ostreococcus* sp. clades C (1 strain), D (1 strain), and A (3 strains), *Bathycoccus* sp., and *Micromonas* sp. were chosen (see Table S3 in the supplemental material) from various worldwide locations to test viral specificity. The same host specificity was observed for the 20 viruses tested (see Table S4 in the supplemental material). All viruses isolated using *O. lucimarinus* as a host were specific to this strain and did not infect any of the other 7 strains tested (5 *Ostreococcus* spp., 1 *Micromonas* sp., and 1 *Bathycoccus* sp.).

Environmental and metagenomic sequences. Environmental DNA *pol* viral sequences were downloaded from GenBank and added to sequences extracted from metagenomic databases (Materials and Methods) and to the 20 new sequences obtained in this study from *O. lucimarinus*. We added some representative viral sequences of *Ostreococcus* and *Micromonas* isolated from a previous study (4) and added a *Bathycoccus* virus sequence as an outgroup to build a phylogenetic tree. This phylogeny clearly splits the sequences into two groups, one containing *Ostreococcus* viruses and the other containing *Micromonas* viruses (Fig. 1). Within the *Ostreococcus* virus clade, viruses from *O. lucimarinus* all cluster together, and 8 environmental sequences can be identified as probable *Ostreococcus* (4 from GenBank and 4 from metagenomes). The geographic origins of all sequences from the *Ostreococcus* virus group obtained in this study are heterogeneous, containing sequences from the North and South Pacific, North Atlantic (East and West), English Channel, and Mediterranean Sea (see Table S3 in the supplemental material).

DISCUSSION

In the present study, we used a host-virus system approach with the aim of isolating new viruses on one host using water samples from worldwide geographic locations. Five main results were obtained. First, viruses from *O. tauri* were only found within the Mediterranean Sea, whereas *O. lucimarinus* viruses were widely distributed (Atlantic, Pacific, Mediterranean Sea, and English Channel). Second, the DNA *pol* sequences of *O. lucimarinus* viruses exhibit a high genetic variability and they did not appear to cluster according to a geographical repartition. Third, the latter sequences recovered from distant oceans form a distinct clade of viruses within the genus *Prasinovirus*. Fourth, sequences with high similarities to *Ostreococcus* viruses can be found within metagenomics data and environmental sequences from different geographic locations. Five, viruses appeared to be highly specific to the host strain from which they were isolated.

In the present study, no *O. tauri* viruses were detectable in samples from 6 sites from different oceans or from one lagoon sample (Moorea, South Pacific). Viruses of *O. tauri* were isolated within the Gulf of Lion near the location where *O. tauri* was originally detected (16). In a previous study, we observed a high abundance of *O. tauri* viruses in a coastal Mediterranean lagoon (Bellec et al., submitted). These results suggest that *O. tauri* viruses may be limited to the Mediterranean Sea and

preferentially within lagoons. Since the first isolation of *O. tauri*, isolates of *Ostreococcus* spp. have been cultured from many oceanic regions (22, 36), and their population density is high in coastal lagoons (3), but oceanic populations appear to be sporadic, with lower densities (100 cells ml⁻¹) (33; Bellec et al., submitted), except during short bloom periods in the North Pacific or North Atlantic with high cell densities (10⁵ cells ml⁻¹) (15, 34). *Ostreococcus* cell density off the Spanish Mediterranean coast (100 km from the coastal lagoon) was close to zero, except for one period at 50 cells ml⁻¹ (56). All of these studies used flow cytometry, denaturing gradient gel electrophoresis (DDGE), or quantitative PCR (qPCR) to assess cell density of the genus *Ostreococcus* but could not discriminate between strains such as *O. tauri* or *O. lucimarinus*. It would thus be informative to investigate coastal lagoons on a worldwide scale.

We found that viruses of *O. lucimarinus* were widely dispersed on a global scale. They were detected within a coastal lagoon in the Mediterranean Sea, displaying a high host specificity, but surprisingly strains similar to the host (*O. lucimarinus*) have not yet been found in cultures from Mediterranean samples. However, environmental sequences (52) show that diverse clade A strains (close to *O. lucimarinus*) are common in the western part of Mediterranean, i.e., in the nutrient-rich waters from the Morocco upwelling, the Strait of Gibraltar, and the Algerian Basin, in surface waters, although these authors did not specifically test water from lagoons. The simplest interpretation of these results is that clade A strains such as *O. lucimarinus* are ubiquitous (although more difficult to detect in cultures because the growth conditions for them may not be optimal), so their viruses are present everywhere, whereas clade C strains, such as *O. tauri*, may be easier to culture but more limited in their distribution to lagoons and coastal areas, so that their viruses in oceanic waters become diluted beyond the limits of detection by our method. This effect may be enhanced by the nature of the Mediterranean basin, with very limited contact with oceanic waters (the Suez Canal and Strait of Gibraltar), and its net water intake since its evaporation rate exceeds freshwater inputs.

Virion dispersal may also play a role in the wide distribution observed here for viruses of *O. lucimarinus*. Although the lifetime of viral particles in nature must be finite, we do not believe that virion viability plays a major role in reducing viral particle densities, since suspensions of viruses in the lab in seawater are stable for >3 years at 4°C and >1 year at 20°C (unpublished data). If these results could be confirmed in the field, that would suggest that viruses can move between biomes following oceanic currents without the need of a host (41). However, in the environment, other factors such as UV, grazing, or degradation by organics may act on viral decay (5, 54), which may be faster under natural conditions. In the sea, loss of viable virions by their erroneous nonspecific binding to related host phytoplankton thus seems a more plausible hypothesis. More information about the distribution of host species and experimental work on the specificity of viral particle binding is necessary to investigate the impact of nonspecific binding, but must await the availability of more complete metagenomic data since the above observations highlight that PCR-based and culture-based techniques give only an incomplete glimpse of species' diversity.

The phylogenetic analysis indicated that all *O. lucimarinus* viruses formed a clade within *Prasinovirus*. There is not a clear geographical distribution, and it is remarkable that viral strains from the South Pacific are closely related to sequences from the English Channel (>95% identity) and from the Mediterranean Sea (>90% identical). However, these three samples were all taken from coastal sites, and their relatedness might reflect a similarity in their host communities. The 20 viruses of *O. lucimarinus* revealed that isolates from one sample site can be as diverse as between isolates from different oceans. For example, viruses from the Mediterranean Sea exhibit a nucleotide divergence of 10.84%, while the divergence between Mediterranean and South Pacific samples is 9.57%. Similar results have been observed for viruses from the prasinophyte *Micromonas*, in which some isolates of *M. pusilla* virus from widely separated geographical locations were more similar than isolates from a single location (12, 13). All of these results suggest that prasinoviruses (or at least *Ostreococcus* and *Micromonas* viruses) are diverse and widely distributed and that genetically distinct isolates can coexist at a single geographic location. Cottrell and Suttle (13) proposed the hypothesis that coexistence of genetically diverse viruses at the same site suggests that competition among these viral strains is limited. This hypothesis may be true for *Micromonas* viruses that exhibit different patterns of host specificity. A previous study showed that two *Micromonas* viruses display clear differences in their infection process. There was no relationship between susceptibility and geographic provenance of the host, nor with the season of collection of the host and the virus (57). In contrast, other studies on *Micromonas pusilla* (9, 40) reported susceptibility only in host strains from the same location of their virus (for DNA and RNA viruses). This pattern was also observed in *Heterosigma akashiwo* viruses (29, 49), different host isolates showing different viral infection characteristics can coexist during a bloom (50). The specificity of *Phycodnaviridae* was also investigated in several host-virus systems: in *Emiliania huxleyi* viruses that exhibited a similar host range for 10 virus isolates (42); in *Chrysochromulina brevifilum* viruses that lysed only 2 out of 10 *Chrysochromulina* host species (47); and, recently, in a virus of *Ostreococcus tauri* (OtV5), that showed strict host-strain specificity (18). Viral specificity is likely to play an important role in the host communities (14) because differences in host range imply different viral pressures on host genetic diversity.

This study presents primary results from a host-virus system on a global scale. However, the lack of knowledge about host ecology (cell density and susceptibility to viruses) and the few data currently available on their viruses emphasize the need for a deeper exploration of these associations in the future, including complete viral sequences to gain insight into those gene functions that may be involved in specificity and in-depth metagenomic analyses to form a more comprehensive nonbiased inventory of all the phycodnaviruses and unicellular eukaryotes present at various geographic sites. The lack of detection of *O. tauri* viruses in the oceans highlights that, even among life's smallest life forms, the spatial heterogeneity of oceanic phytoplankton requires further exploration.

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