RNA viruses in the sea

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Received 31 March 2008; revised 29 July 2008; accepted 21 August 2008.
First published online 26 September 2008.
DOI:10.1111/j.1574-6976.2008.00132.x

Editor: Cornelia Büchener-Osmond

Keywords
RNA virus; virioplankton; virus diversity; marine virus; virus ecology; aquaculture.

Abstract
Viruses are ubiquitous in the sea and appear to outnumber all other forms of marine life by at least an order of magnitude. Through selective infection, viruses influence nutrient cycling, community structure, and evolution in the ocean. Over the past 20 years we have learned a great deal about the diversity and ecology of the viruses that constitute the marine virioplankton, but until recently the emphasis has been on DNA viruses. Along with expanding knowledge about RNA viruses that infect important marine animals, recent isolations of RNA viruses that infect single-celled eukaryotes and molecular analyses of the RNA virioplankton have revealed that marine RNA viruses are novel, widespread, and genetically diverse. Discoveries in marine RNA virology are broadening our understanding of the biology, ecology, and evolution of viruses, and the epidemiology of viral diseases, but there is still much that we need to learn about the ecology and diversity of RNA viruses before we can fully appreciate their contributions to the dynamics of marine ecosystems. As a step toward making sense of how RNA viruses contribute to the extraordinary viral diversity in the sea, we summarize in this review what is currently known about RNA viruses that infect marine organisms.

Introduction to marine viruses

Marine viruses and their consequences
Viruses are an integral part of the marine ecosystem. As we assume to be true of all life on our planet, every living thing in the ocean appears to be susceptible to disease and death caused by viral infections. Although viruses cannot replicate autonomously, they outnumber all forms of cellular life in the oceans by roughly an order of magnitude (Maranger & Bird, 1995). The concentration of free virions in seawater (i.e. the marine virioplankton) is typically billions to tens-of-billions per liter at the surface (Bergh et al., 1989; Wommack & Colwell, 2000). The concentration of virioplankton does decline with depth, but they appear to be ubiquitous and are found deep in the ocean’s interior (Cochlan et al., 1993; Hara et al., 1996; Ortmann & Suttle, 2005), yielding an estimated total abundance of $> 10^{30}$ in the sea (Suttle, 2005).

The virioplankton are not only numerous, but also extraordinarily diverse, both morphologically (Frank & Moebus, 1987; Weinbauer, 2004) and genetically (Edwards & Rohwer, 2005). Analyses of environmental shotgun clone libraries suggest that there may be a few thousand to perhaps 10 000 viral genotypes in samples from individual marine habitats (Breitbart et al., 2002; Bench et al., 2007). When seawater from four distinct oceanic regions was analysed, it was estimated that more than 50 000 genotypes were present among the samples (Angly et al., 2006).

Viruses can be a major source of disease and mortality for marine life. Epidemic infections have caused occasional mass mortalities of marine mammals (Geraci et al., 1982; Jensen et al., 2002) and fish (Skall et al., 2005), and some data suggest that viruses might have a role in coral bleaching (Wilson et al., 2001; Davy et al., 2006; Lohr et al., 2007). Marine viruses can even influence human health and prosperity. Epidemic viral infections of commercially exploited marine animals such as salmonids, shrimp, abalone, and oysters (Hasson et al., 1995; Friedman et al., 2005; McLoughlin & Graham, 2007), for example, can cause significant economic hardship. The ocean is also a reservoir of viruses that can infect terrestrial organisms (Sano et al., 2004), including humans (Smith et al., 1998a, b).
Viruses have been implicated in the natural termination of plankton blooms, some of which occur on a massive scale. For example, the marine coccolithophorid *Emiliania huxleyi* is capable of forming blooms in temperate waters that cover upwards of 10 000 km², and viruses have been identified as the primary agent of *E. huxleyi* bloom termination in several instances (Bratbak *et al.*, 1993, 1996; Brussaard *et al.*, 1996; Jacquet *et al.*, 2002; Wilson *et al.*, 2002). Viruses have also been found in association with bloom termination events in other systems (Nagasaki *et al.*, 1994; Tarutani *et al.*, 2000; Tomaru *et al.*, 2004a; Baudoux *et al.*, 2006).

Viruses play an important role in the cycling of nutrients in the ocean through their infection and lysis of marine microorganisms. The overall effect of this viral activity is to augment the rate of movement of nutrients from particulate organic matter to dissolved organic matter, diverting nutrients from higher trophic levels back into the microbial fraction (Fuhrman, 1992; Suttle, 2007). Viruses also appear to promote diversity in planktonic communities (Van Hannen *et al.*, 1999; Weinbauer & Rassoulzadegan, 2004; Bouvier & del Giorgio, 2007). One explanation is that, because viral infections are often host specific and the spread of an infection is density dependent, viruses tend to 'kill the winner' (Thingstad *et al.*, 1993) and prevent the dominance of a single species. The release of diverse organic substrates from lysed cells that can be readily used by other bacteria appears to be a complementary process promoting diversity (Middelboe *et al.*, 2003).

In addition to controlling the balance among existing species, viruses create new diversity by driving evolution of life on land and in the sea (Villarreal, 2005). The influence of viruses on evolutionary processes is particularly evident among the bacteria. Analyses of microbial genome sequences show that viruses actively modify bacterial genomes and that gene exchange between virus and host is a common occurrence (Canchaya *et al.*, 2003a, b; Casjens, 2003).

**The contribution of RNA viruses to the viroplankton**

Since the first direct counts of viruses in seawater revealed their extraordinary abundance nearly two decades ago, the question of what contribution RNA viruses make to the viroplankton has been virtually ignored. A number of lines of indirect evidence have been used to argue that most of the viruses constituting the marine viroplankton have DNA genomes (Steward *et al.*, 1992; Weinbauer, 2004). The actual numerical contribution of RNA viruses to the marine viroplankton has remained rather poorly constrained, however, because of methodological limitations. Routine staining procedures for electron microscopy do not distinguish the nucleic acid content of viral particles (Fig. 1), while flow cytometry and epifluorescence microscopy (Fig. 2) appear to lack the sensitivity, at present, to accurately enumerate viruses with small genomes containing either RNA or DNA (Brussaard *et al.*, 2000; Tomaru & Nagasaki, 2007).
Despite uncertainties about their abundance, RNA viruses are diverse and ecologically important. RNA viruses of every major classification (single- and double-stranded, positive- and negative-sense), and which infect a diverse range of host species, have been isolated from the sea (Table 1). Diseases caused by RNA viruses can have devastating effects on populations of aquatic animals. The causes and consequences are best understood for farmed aquatic animals where factors such as high-density culture, substandard environmental conditions and nonregulated movement of animals can increase the risk and incidence of viral disease outbreaks. Molecular biology and genomic methods are rapidly advancing the study of host and pathogen genomes and the molecular mechanisms involved in host–pathogen interactions in marine fish and shellfish (e.g. von Schalburg et al., 2005; Purcell et al., 2006; Rattanarojpong et al., 2007; Poisabeiro et al., 2008; Rise et al., 2008). These studies promise to aid in the development of new diagnostics for identifying viral pathogens in animal tissues and water samples, novel approaches and models for studying the dissemination and evolution of marine RNA viruses, and tools such as vaccines and therapeutics for preventing outbreaks of viral diseases in populations of farmed aquatic animals. Because they are of immediate practical concern, RNA viruses infecting commercially exploited shellfish and fish species, and those infecting marine mammals, have already received considerable attention. In contrast, most of what we know about RNA viruses infecting unicellular marine plankton has been learned only in the past 5 years. However, this work is clearly demonstrating important linkages between host species dynamics and viral abundances in natural environments.

**Purpose and scope of the review**

A number of reviews that cover various aspects of marine viruses have been published in the last 10 years (Fuhrman, 1999; Wilhelm & Suttle, 1999; Wommack & Colwell, 2000; Brussaard, 2004; Weinbauer, 2004; Suttle, 2005, 2007; Munn, 2006; Danovaro et al., 2008; Nagasaki, 2008). None of these has focused exclusively on RNA viruses. The time for such a review seems appropriate for two reasons. The first is that there have been a number of significant discoveries about RNA viruses in the sea over the past 5 years that are worth highlighting. The second is that environmental genomic and transcriptomic analyses of marine microbial communities are gaining popularity and as we begin to mine the extraordinary genetic diversity of RNA viruses in the ocean, it is worth our while to summarize what we know about the kinds of viruses we may expect to find there. To this end, we have compiled information on RNA viruses that are known to infect organisms that spend a significant amount of time on or in the ocean. We then review the recent discoveries about marine RNA viruses that have been made using cultivation-independent techniques. It is becoming clear that, at this time, most of the RNA virus diversity in the sea is unknown.

**Marine RNA virus–host systems**

RNA viruses can be subdivided into those with double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) genomes. The ssRNA viruses can be further subdivided into those that package positive-sense (coding) and negative-sense (noncoding) RNA molecules into the viral particles. To illustrate the diversity and importance of RNA viruses in the sea, here we review the present knowledge of RNA viruses in the sea, organized first by virus taxonomic assignment and then by host type (when appropriate) within the specific virus taxonomic groups.

**Positive-sense ssRNA viruses**

The positive-sense ssRNA viruses represent the most diverse collection of defined taxonomic groups of any virus genome type with more than 20 defined families and numerous other unclassified genera (Fauquet et al., 2005). Viruses from at least eight of the known families infect marine organisms, although many viruses remain unclassified.

**Picornavirales**

The order Picornavirales comprises a group of virus families and unassigned genera that are related to the Picornaviridae and that share certain properties (Le Gall et al., 2008). Viruses in this order are known to be present in a broad range of marine environments and host species (Mari et al., 2002; Culley et al., 2003; Lang et al., 2004; Culley et al., 2006, 2007; Shirai et al., 2006; Takao et al., 2006; Culley & Steward, 2007), and include pathogens of marine animals. We include in this section several viruses that are not yet officially classified within the Picornavirales, but that seem most likely to belong to this order.

**Protistan hosts**

The first report of an RNA virus infecting a marine protist appeared only 5 years ago (Tai et al., 2003). Since then, several more protistan RNA viruses have been isolated and molecular surveys of the environment suggest that many more have yet to be discovered. Protists occur throughout the water column (Cho et al., 2000; Countway et al., 2007; Fukuda et al., 2007; Not et al., 2007) but are especially abundant in surface waters where they can account for c. 50% of the biomass (Caron et al., 1995). The marine protists are not only abundant, but extraordinarily diverse (Adl et al., 2005; Keeling et al., 2005), and ecologically and economically important (Anderson et al., 1998). The limited
<table>
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<td><strong>Positive-sense ssRNA viruses</strong></td>
<td><strong>Marnaviridae (Picornavirales)</strong></td>
<td>HaRNAV</td>
<td>Heterosigma akashiwo (protist)</td>
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<td></td>
<td><strong>Dicistroviridae (Picornavirales)</strong></td>
<td>TSV</td>
<td>Penaeid shrimp</td>
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<td></td>
<td><strong>Picornaviridae (Picornavirales)</strong></td>
<td>SePV-1</td>
<td>Ringed seal (Phoca hispida)</td>
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<td></td>
<td><strong>Caliciviridae</strong></td>
<td>SMSV</td>
<td>Marine mammals, humans, invertebrates, fish</td>
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<td></td>
<td></td>
<td>WCV</td>
<td>Walrus (Odobenus rosmarus)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCV</td>
<td>California sea lion (Zalophus californianus), Steller sea lion (Eumetopias jubatus)</td>
</tr>
<tr>
<td></td>
<td><strong>Unidentified</strong></td>
<td></td>
<td>Fish, white tern (Gygis alba)</td>
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<td></td>
<td><strong>Nodaviridae</strong></td>
<td>BFNNV, ACNNV, DIEV, JFNNV, LcEV, RGNV, SJNNV, TPNNV</td>
<td>Numerous fish species [e.g. barfin flounder (Verasper moseri), Atlantic cod (Gadus morhua), tiger puffer (Takifugu rubripes), Japanese flounder (Paralichthys olivaceus)]</td>
</tr>
<tr>
<td></td>
<td><strong>PnNV</strong></td>
<td></td>
<td>Penaeus vannamei (shrimp)</td>
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<td></td>
<td><strong>Togaviridae</strong></td>
<td>SPDV/SAV1</td>
<td>Atlantic salmon (Salmo salar)</td>
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<td></td>
<td></td>
<td>NSAV/SAV3</td>
<td>Atlantic salmon, rainbow trout (Oncorhynchus mykiss)</td>
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<td></td>
<td></td>
<td>SDV/SAV2</td>
<td>Rainbow trout</td>
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<td></td>
<td></td>
<td>SES</td>
<td>Louse (Lepidopthirus macrorhini), southern elephant seal (Mirounga leonina)</td>
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<td></td>
<td><strong>Roniviridae</strong></td>
<td>YHV</td>
<td>Various species of shrimp, prawns and krill</td>
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<tr>
<td></td>
<td><strong>Flaviviridae</strong></td>
<td>TYUV</td>
<td>Tufted puffin (Fratercula cirrhata), common murre (Uria aalge), thick-billed murre (U. lomvia)</td>
</tr>
<tr>
<td></td>
<td><strong>Unclassified</strong></td>
<td>HcRNAV</td>
<td>Heterocapsa circularisquama (protist)</td>
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<td></td>
<td></td>
<td>RsRNAV</td>
<td>Rhizosolenia setigera (protist)</td>
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<td></td>
<td></td>
<td>CtenRNAV</td>
<td>Chaetoceros tenuissimus (protist)</td>
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<td></td>
<td>SssRNAV</td>
<td>Aurantiochytrium sp. (protist)</td>
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<tr>
<td></td>
<td></td>
<td>LSNV</td>
<td>Black tiger shrimp (Penaeus monodon)</td>
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<td></td>
<td></td>
<td>CsfRNAV</td>
<td>Chaetoceros socialis (protist)</td>
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<td></td>
<td></td>
<td>06N-58P</td>
<td>Pseudomonas sp.</td>
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<td></td>
<td></td>
<td>JP-A</td>
<td>Unknown (protist?)</td>
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<tr>
<td></td>
<td></td>
<td>JP-B</td>
<td>Unknown (protist?)</td>
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<tr>
<td></td>
<td></td>
<td>SOG</td>
<td>Unknown</td>
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</table>
### Table 1. Continued.

<table>
<thead>
<tr>
<th>Virus classification</th>
<th>Host species</th>
<th>Properties and comments</th>
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<tbody>
<tr>
<td><strong>Negative-sense ssRNA viruses</strong></td>
<td></td>
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</tr>
<tr>
<td>Orthomyxoviridae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISAV</td>
<td>Atlantic salmon, sea trout (<em>Salmo trutta</em>), Arctic char (<em>Salvelinus alpinus</em>), herring (<em>Clupea harengus</em>)</td>
<td>Eight segments (from 0.9 to 2.4 kb) with 12.7 kb total genome; enveloped</td>
</tr>
<tr>
<td>Influenza A</td>
<td>Marine mammals [e.g. harbor seal (<em>Phoca vitulina</em>), pilot whales (<em>Globicephala melaena</em>), many birds, humans]</td>
<td>Eight segments (from 0.9 to 2.4 kb) with 13.6 kb total genome; enveloped</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDV</td>
<td>Various marine mammals</td>
<td>Serious impacts on seal populations</td>
</tr>
<tr>
<td>CeMV</td>
<td>Various marine mammals</td>
<td></td>
</tr>
<tr>
<td>NDV</td>
<td>Common murre</td>
<td></td>
</tr>
<tr>
<td>Rhabdoviridae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VHSV, IHNV, HIRRV, SHRV, SVCV</td>
<td>Many fish species [e.g. Atlantic cod, coho salmon (<em>Oncorhynchus kisutch</em>), Pacific cod (<em>Gadus macrocephalus</em>), Pacific herring (<em>Clupea pallasii</em>), penaeid shrimp</td>
<td>11–12 kb genome, polycistronic; Fig. 6; enveloped</td>
</tr>
<tr>
<td>DRV</td>
<td>White-beaked dolphin (<em>Lagenorhynchus albirostris</em>)</td>
<td>Possibly broad host range</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td></td>
<td></td>
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<tr>
<td>MoV</td>
<td>Penaeid shrimp</td>
<td>Three segments, 11–19 kb total genome; enveloped</td>
</tr>
<tr>
<td>Avalon, Farallon, Zaliv Terpeniya</td>
<td>Common murre, thick-billed murre, tufted puffin, Atlantic puffin (<em>Fratercula arctica</em>)</td>
<td>Transmission by invertebrate vectors at breeding colonies</td>
</tr>
<tr>
<td><strong>dsRNA viruses</strong></td>
<td></td>
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<tr>
<td>Reoviridae</td>
<td></td>
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</tr>
<tr>
<td>13p2</td>
<td>American oyster (<em>Crassostrea virginica</em>)</td>
<td>11 segments, 24 kb total genome</td>
</tr>
<tr>
<td>CSRV</td>
<td>Chum salmon (<em>Onchorhynchus keta</em>)</td>
<td>11 segments, 24 kb total genome</td>
</tr>
<tr>
<td>P</td>
<td>Mediterranean swimming crab (<em>Macropius depurator</em>)</td>
<td>12 segments, 23 kb total genome</td>
</tr>
<tr>
<td>W2</td>
<td>Mediterranean shore crab (<em>Carcinus mediterraneus</em>)</td>
<td>12 segments, 24 kb total genome</td>
</tr>
<tr>
<td>MCRV</td>
<td>Mud crab (<em>Scylla serrata</em>)</td>
<td>13 segments, 26.9 kb total genome</td>
</tr>
<tr>
<td>MpRV</td>
<td>Micromonas pusilla (protist)</td>
<td>11 segments, 25.6 kb total genome</td>
</tr>
<tr>
<td>Bauline virus, Kemerovo virus, Okhotskiy virus</td>
<td>Common murre, thick-billed murre, tufted puffin, Atlantic puffin, razorbill (<em>Alca torda</em>)</td>
<td>Species Great Island virus</td>
</tr>
<tr>
<td>Birnaviridae</td>
<td></td>
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<tr>
<td>IPNV</td>
<td>Many fish species [e.g. Atlantic salmon, red sea bream (<em>Pagrus major</em>), American plaice (<em>Hippoglossoides platessoides</em>), Atlantic cod], mollusks [e.g. Japanese pearl oyster (<em>Pinctada fucata</em>), jackknife clam (<em>Sinonovacula constricta</em>)]</td>
<td>3.1 and 2.7 kb bi-segmented genome; Fig. 6</td>
</tr>
<tr>
<td>Retroviridae</td>
<td></td>
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<tr>
<td>RV lemon shark</td>
<td>Lemon shark (<em>Negaprion brevirostris</em>)</td>
<td>Sequences amplified by PCR from fish genome</td>
</tr>
<tr>
<td>RV puffer fish</td>
<td>Puffer fish (<em>Fugu rubripes</em>)</td>
<td>Sequences amplified by PCR from fish genome</td>
</tr>
<tr>
<td>Unclassified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMNV</td>
<td>Penaeid shrimp</td>
<td>7.6 kb genome; Fig. 6</td>
</tr>
</tbody>
</table>

†Most closely related to family Totiviridae
High mortalities in farmed shrimp
characterizations of marine RNA virophagons reported to date suggest that the majority infect protists. This may not be surprising if we consider that the vast majority of eukaryotic diversity on the planet (Keeling et al., 2005) is in fact comprised of aquatic protists.

The first RNA virus reported to infect a protist was HaRNAV, a ssRNA virus that infects the unicellular photosynthetic marine flagellate Heterosigma akashiwo (Tai et al., 2003). The population dynamics of H. akashiwo are of special economic interest as blooms of this alga are responsible for extensive fish kills worldwide, affecting the aquaculture industry in particular (Smyda, 1998). Viruses appear to contribute to the termination of blooms of this organism (Nagasaki et al., 1994; Tarutani et al., 2000), and, in addition to HaRNAV, there are a number of different viruses known that infect H. akashiwo (Nagasaki & Yamaguchi, 1997; Lawrence et al., 2001, 2006). HaRNAV was isolated from the southern Strait of Georgia, BC, Canada where H. akashiwo produces yearly spring blooms. It has a nonenveloped,icosahedral capsid that is c. 25 nm in diameter, and contains a genome of c. 8.6 kb (Tai et al., 2003; Lang et al., 2004). Addition of HaRNAV to a growing culture of susceptible H. akashiwo causes lysis of the culture within 7 days or less (Tai et al., 2003), with the first evidence of cell death appearing after 1 day (Lawrence et al., 2006). Characteristics of HaRNAV infection include formation of viral crystalline arrays in the cytoplasm of infected cells, swelling of the endoplasmic reticulum (ER) and development of ER-associated vesicles, and vacuolation of the cytoplasm (Tai et al., 2003) (Fig. 3). Some features of this pathology are remarkably conserved with what is seen in other positive-

Fig. 3. Replication of HaRNAV inside Heterosigma akashiwo. Note the large numbers of viral particles in the cytoplasm, the vacuolation of the cytoplasm and in fibrils in the vacuoles (e.g. black arrow), and the vesicles associated with the ER (e.g. white arrows). The transmission electron micrograph of a negatively stained, sectioned HaRNAV-infected cell was taken c. 72 h postinfection. Scale bar, 500 nm. Modified from Tai et al. (2003) with permission of the authors and Blackwell Publishing.

Fig. 4. Genome organizations of the cultured positive-sense ssRNA viruses that infect marine protists. The identified ORFs within the genome sequences are indicated as boxes. Regions that contain recognized conserved ssRNA virus domains are indicated and shaded in colour as follows. H: RNA helicase, orange; RdRp: RNA-dependent RNA polymerase, purple; CP: capsid protein(s), yellow; P: protease, green. The genome of SssRNAV contains a small ORF at the 3’ end that is expressed as a subgenomic RNA during replication (labeled with the number 3). The genomes are approximately to scale as indicated by the bar. Maps were drawn using information from previous publications (Lang et al., 2004; Nagasaki et al., 2005; Shirai et al., 2006; Takao et al., 2006).
viruses classified in the family *Dicistroviridae*, including cricket paralysis virus (CrPV) for which a crystal structure has been determined (Tate et al., 1999). Also, the HaRNAV organization of the structural protein subunits within the larger (structural portion of the) polyprotein is the same as found in the family *Dicistroviridae* (Lang & Suttle, 2008). Despite these shared characteristics and the established phylogenetic relationships, a large part of the HaRNAV genome has no recognizable similarity to any other known sequences, viral or otherwise. More work needs to be done to understand the function of most of the genome and the biology of this virus.

A culture-independent reverse transcriptase-PCR (RT-PCR) approach that targeted picorna-like virus RdRp genes in coastal British Columbia marine virus communities produced a number of distinct RdRp sequences that were very similar to HaRNAV (Culley et al., 2003). While different from the sequenced strain of HaRNAV, these sequences did have a high level of identity with HaRNAV and likely represent different strains of this virus (Lang & Suttle, 2008). This also showed that HaRNAV is a persistent virus in these waters (Culley et al., 2003).

Diatoms are another major group of protists in marine systems, with a large diversity in known species [>10,000 recognized and >100,000 estimated (Norton et al., 1996)] and a diverse array of possible lifestyles including benthic, planktonic, or in specific associations with plants or animals (Graham & Wilcox, 2000). They are thought to be the most abundant aquatic eukaryotic organisms and make a large contribution to global primary production and biogeochemical cycling (Graham & Wilcox, 2000). Therefore, viruses infecting these organisms could have significant effects on diatom population dynamics and these processes. The first virus isolate found to infect a diatom was the positive-sense ssRNA virus RsRNAV, which infects *Rhizosolenia setigera* (Nagasaki et al., 2004b), a diatom common in temperate coastal waters. This diatom forms blooms that, while not reported to have directly toxic effects on other organisms, do have an economic impact by reducing nutrient availability for commercially important seaweed species (Nagasaki et al., 2004b).

RsRNAV has similarities to HaRNAV, with an icosahedral, nonenveloped, c. 32 nm diameter capsid, and replication in the cytoplasm that shows large numbers of particles (burst size estimated at >3000) being produced in infected cells (Nagasaki et al., 2004b). Addition of the virus to a culture of *R. setigera* leads to a decrease in host cell numbers in a culture after 2 days coincident with virus appearance in the culture medium (Nagasaki et al., 2004b). The positive-sense 8.9-kb genome has a poly(A) tail and encodes two polyproteins (Shirai et al., 2006). The 5′ ORF-1 is predicted to encode the nonstructural proteins including putative RNA helicase and RdRp domains, and the 3′ ORF-2 is predicted to encode the major structural proteins (Fig. 4); the N-terminus has been sequenced for one of the structural proteins (Shirai et al., 2006). Use of the conserved domains from the RsRNAV sequence for phylogenetic analyses have shown that it clearly has a relationship with the classified protist-infecting virus HaRNAV (family *Marnaviridae*) as well as other marine and nonmarine viruses, but that it likely represents a different family within the *Picornavirales* (Fig. 7), and this is further supported by the differences in genomic organization (Fig. 4; Culley et al., 2006, 2007; Shirai et al., 2006; Lang & Suttle, 2008).

Diatoms have silica frustules, and this would appear to be an effective barrier against viral infection. However, the pores in the frustule of *R. setigera* are larger than RsRNAV particles and represent a potential entry point for this virus (Nagasaki et al., 2004b). If viral particles must traverse the frustule pores in order to infect a diatom, this would represent a major selective pressure for diatom viruses to be small.

Another diatom-infecting ssRNA virus, CtenRNAV, was recently discovered in *Chaetoceros tenuissimus* Meunier (Shirai et al., 2008). The CtenRNAV particles are icosahedral with a diameter of c. 31 nm. A large number of virus particles are visible in the cytoplasm of infected cells, where they can form crystalline arrays, and the burst size is estimated to be very large, at c. 10⁴ per cell. The yield of viral particles from an infected culture is also extraordinary, at c. 10¹⁰ infectious particles per mL. The genome sequence apparently contains two ORFs with a similar organization to that found in the diatom virus RsRNAV (Fig. 4), and a phylogenetic analysis of RdRp sequences shows the virus to be most closely related to RsRNAV (Shirai et al., 2008) making it likely that it is also a member of the order *Picornavirales*. Interestingly, the viral particles appear to contain two ssRNA molecules with one representing the complete genome (9431 nt) while the nature of the second smaller RNA molecule (4.3 kb) is currently unknown (Shirai et al., 2008). A different ssRNA virus, CsfrRNAV, has been discovered that infects a diatom in the same genus, *Chaetoceros socialis* f. *radians* (Y. Tomaru, pers. commun.); more details about the exact nature of this virus are forthcoming.

As well as infecting ecologically important eukaryotic phytoplankton, an RNA virus that infects a heterotrophic protist has been isolated. SsRNAV is a ssRNA virus with a nonenveloped, spherical, c. 25 nm diameter capsid that infects the thaustochrytid originally identified as *Schizochytrium* sp. (Takao et al., 2005) but subsequently reclassified as *Aurantiochytrium* sp. (Yokoyama & Honda, 2007). Thraustochytrids are osmotrophic marine fungoid protists found in a wide-range of aquatic habitats, where they serve as important decomposers (Kimura et al., 1999) and they can make up a significant proportion of the biomass, approaching 50% in some locations (Naganuma et al.,...
that, unlike the replication of multiple subgenomic RNAs during replication suggests that ORF1 encoding a protein important for replication of this ORF does indeed encode a protein important for replication of the virus (Takao et al., 2006). The production of multiple subgenomic RNAs during replication suggests that, unlike the *Dicistroviridae* that have a similar dicistronic genome organization, SssRNAV does not use IRES elements for translation of the 3′ ORFs (Takao et al., 2006).

Although SssRNAV shares some characteristics with HaRNAV and RsRNAV (Fig. 7), phylogenetic analyses and comparisons of genome architecture strongly support the placement of SssRNAV outside established families of RNA viruses although it is likely a member of the order *Picornavirales* (Takao et al., 2006; Culley et al., 2007; Lang & Suttle, 2008).

**Invertebrate hosts**

Taura Syndrome Virus (TSV) is a well-known shrimp pathogen within the family *Dicistroviridae* (Kiatpathomchai et al., 2007). The principal hosts for this disease are the two penaeid shrimps the Pacific White Shrimp (*Litopenaeus vannamei*) and the Pacific Blue Shrimp (*Litopenaeus stylirostris*). Owing to the devastating impact of Taura syndrome disease on penaeid shrimp aquaculture since the disease emerged in Ecuador in 1992 (Brock et al., 1997; Robles-Sikisaka et al., 2001), the causative agent of Taura syndrome disease (TSV) has received considerable research attention over the past decade. The viral etiology of this disease was demonstrated in 1995 (Hasson et al., 1995) (Fig. 5), and TSV was characterized in detail in 1997 (Bonami et al., 1997). TSV has nonenveloped, icosahedral particles c. 32 nm in diameter and a genome that consists of a linear positive-sense ssRNA (Bonami et al., 1997). The 10.2-kb genome has two ORFs with ORF1 encoding nonstructural proteins such as helicase, protease, and RdRp and ORF2 encoding the structural or capsid proteins (Fig. 6) (Mari et al., 2002). Molecular phylogenetic analysis based on RdRp sequence alignments and other biological properties show that TSV belongs in the *Cripavirus* genus (Christian et al., 2005). TSV is the first member of this genus known to infect a noninsect host (Mari et al., 2002). Characterization of TSV gene sequences has lead to the development of RT-PCR-based molecular diagnostics (Dhar et al., 2002) and new strategies for combating the virus. For example, transgenic shrimp that stably express an antisense transcript designed from the TSV coat protein (CP) gene have enhanced resistance to TSV (Lu & Sun, 2005). The molecular mechanism involved in this antisense RNA-based antiviral strategy may be the targeted degradation of the resulting dsRNA duplexes between TSV sense CP transcript and the transgene antisense CP transcript by dsRNA nucleases, and/or a nonspecific, sequence-independent, antiviral response elicited by dsRNA (Robalino et al., 2004; Lu & Sun, 2005). TSV has been shown to be capable of infecting cultured human and monkey cells (Audelo-del-Valle et al., 2003). This raises the possibility that TSV could be transmissible to humans from shrimp and if so, TSV-infected shrimp could be a threat to public health (Audelo-del-Valle et al., 2003).

**Fig. 5.** TSV of shrimp. (a) Electron micrograph of negatively stained TSV particles. The arrow points to an empty particle. Scale bar, 32 nm. (b) *Penaeus vannamei* showing gross external melanized cuticular lesions characteristic of TSV infection (4 days after infection). (c) Histological sections of *P. vannamei* cuticular epithelium showing characteristic TSV tissue lesions. The lesions show multiple pale to darkly staining spherical inclusion bodies of varying diameters (arrowheads). Scale bar, 20 μm. Reproduced from Hasson et al. (1995) with permission of the authors and Inter-Research.
Mammalian hosts

A picornavirus was recently isolated from ringed seals (Phoca hispida) that appears to define a new genus within the family Picornaviridae (Kapoor et al., 2008). The virus, seal picornavirus 1 (SePV-1), has a monocistronic genome of 6693 nts and shows the closest phylogenetic association with the duck hepatitis virus (DHV) group and the Par echovirus genus, but is distinct from these (Kapoor et al., 2008). Assays, using RT-PCR, on cell cultures that had been inoculated with material from a collection of 108 seals killed between 2000 and 2002 in the Beaufort Sea, showed 7.4% were positive for SePV-1 (Kapoor et al., 2008).

Caliciviridae

Viruses in the family Caliciviridae are known to infect a variety of marine species including mammals, birds, fish, and invertebrates (Van Bressem et al., 1999; Smith, 2000). This includes the walrus calicivirus (WCV) recently isolated from walrus (Odobenus rosmarus) feces, and this virus appears closely related to most of the other marine caliciviruses that have been described (Ganova-Raeva et al., 2004). Most, if not all, marine caliciviruses are in the genus Vesivirus (Fig. 6) and grouped together as San Miguel sea lion viruses (SMVs) (Smith, 2000), named for the initial isolation from California sea lions (Zalophus californianus) on San Miguel Island (CA) (Smith et al., 1973). SMVs have been isolated from at least 25 species, including a number of marine mammals, but also invertebrates and fish. The fish opaleye perch (Girella nigricans) (Smith et al., 1980b) and nematode lungworm (Parafilaroides decorus) (Smith et al., 1978) hosts of SMV are thought to have a significant role in virus transmission between individual mammals (Smith et al., 1980a). The concentrations of caliciviruses could be extremely high in some places given the large number of viruses that infected individuals can shed. For example, it is estimated that an infected California gray whale (Eschrictus gibbosus) can shed 10^13 viruses in its feces every day (Smith et al., 1998a). Many marine mammals are migratory and so there is great potential for virus transmission around the world’s oceans by infected individuals. Caliciviruses appear readily able to jump between species, and between marine and terrestrial hosts. There is documented transmission of marine caliciviruses from contaminated fish scraps to domesticated swine (reviewed in Smith, 2000), and a virus that was first found in a captive snake and described as reptilian calicivirus (RCV) (Smith et al., 1986) was subsequently isolated from marine mammals (Barlough et al., 1998). There is also a known instance of transmission of a

Fig. 6. Gene organization in some of the RNA viruses that infect marine vertebrate and invertebrate hosts. ORFs within the genome sequences are indicated as boxes. Regions that contain known or conserved protein domains are indicated and shaded in colour as follows. H: RNA helicase, orange; P: protease, green; RdRp: RNA-dependent RNA polymerase, purple; CP: capsid protein(s), yellow; E or G: envelope glycoprotein(s), pink. In the SAV genome, the region shaded grey and labeled Mt is a methyl transferase. In the HIRRV genome, the genes are labeled: N, nucleocapsid; PhP, phosphoprotein; M, matrix; NV, nonvirion; L, large (= RdRp). The segments A and B are labeled for the IPNV genome; the ORF labeled “S” on segment A encodes a protein of unknown function, VPS. In the IMNV genome the region shaded grey and labeled R is a dsRNA-binding motif (DSRM). The genomes are approximately to scale as indicated by the bar. Maps were drawn using information from previous publications (Duncan et al., 1987; Carter et al., 1992; Mari et al., 2002; Weston et al., 2002; Zhang & Suzuki, 2003; Romero-Brey et al., 2004; Kim et al., 2005; Koopmans et al., 2005; Weaver et al., 2005; Poulos et al., 2006).
calicivirus from prey fish to a white tern (Gygis alba) (Poet et al., 1996). Infection of such migratory species provides clear opportunities for spread of viruses to new locations. There are also known instances of calicivirus transmission from marine mammals to humans.

**Nodaviridae**

Fish nodaviruses, members of the genus Betanodavirus within family Nodaviridae, have a worldwide distribution and pose a serious threat to the farming of marine fish species. Betanodaviruses are the causative agents of viral nervous necrosis (VNN), also called viral encephalopathy and retinopathy (VER). Betanodaviruses are small, non-enveloped, segmented viruses. There are two genome segments, RNA1 that encodes a putative RdRp as well as protein B2 (encoded from the 3′ end of RNA1), and RNA2 that encodes the capsid protein precursor α (Mori et al., 1992). The genus Betanodavirus is divided into eight clades: barfin flounder nervous necrosis virus (BFNNV), Atlantic cod nervous necrosis virus (ACNNV) Dicentrarchus labrax encephalitis virus (DIEV), Japanese flounder nervous necrosis virus (JFNNV), Latest calcarifer encephalitis virus (LcEV), redspotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), and tiger puffer nervous necrosis virus (TPNNV) based on nucleotide sequence comparisons of the variable region (T4) of the capsid protein (Gagne et al., 2004).

Disease caused by nodaviruses has been reported in over 30 species of marine fish including several of economic importance (Munday et al., 2002; Cutrin et al., 2007). Signs of VNN disease include the presence of vacuoles in the brain and retina, and behavioral and swimming disorders that are consistent with neurological damage (Thiery et al., 2004). Betanodavirus sequence has been identified in several species of marine animals including four fish species [Japanese scad (Decapterus maruadsi), marbled rockfish (Sebastiscus marmoratus), threadtail filefish (Stephanolepis cirrhifer) and black scraper (Thamnaconus modestus)] sampled from Japanese waters (Gomez et al., 2004), wild-winter flounder (Pleuronectes americanus) (Barker et al., 2002) and asymptomatic farmed Atlantic cod (Gadus morhua) in Canada (Rise et al., 2008), and apparently healthy marine aquarium fish and invertebrates in Korea (Gomez et al., 2006). In addition, RT-PCR has been used to amplify betanodavirus sequence from seawater in Atlantic halibut (Hippoglossus hippoglossus) rearing facilities in Norway (Nerland et al., 2002; McLoughlin & Graham, 2007). Bird and mammal alphaviruses are transmitted between hosts by hematophagous arthropods such as mosquitoes. Because sea lice may carry salmonid alphavirus (SAV), it is possible that they could act as reservoirs or vectors for the virus (Powers et al., 2001; Weston et al., 2002; Weaver et al., 2005; McLoughlin & Graham, 2007). Alphaviruses replicate in the host cell cytoplasm, and become membrane-bound when they bud through the plasma membrane (McLoughlin & Graham, 2007). Bird and mammal alphaviruses are transmitted between hosts by hematophagous arthropods such as mosquitoes. Because sea lice may carry salmonid alphavirus (SAV), it is possible that they could act as reservoirs or vectors for the virus (Powers et al., 2001; Weston et al., 2002; Karlsen et al., 2006; McLoughlin & Graham, 2007). However, an invertebrate vector of SAV has not yet been identified (McLoughlin & Graham, 2007).

The SAVs include salmon pancreas disease virus [SPDV or SAV1, causing pancreas disease in sea farmed Atlantic salmon (Salmo salar) in Ireland and Scotland], Norwegian salmonid alphavirus [NSAV or SAV3, causing pancreas disease in sea farmed Atlantic salmon and rainbow trout (Oncorhynchus mykiss) in Norway], and sleeping disease virus [SDV or SAV2, causing sleeping disease in freshwater farmed rainbow trout (McLoughlin & Graham, 2007 and references therein). The entire genomes of SPDV (11 919 nt) and SDV (11 900 nt) have been sequenced and compared, revealing that they are 91.1% identical at the nucleotide level and 94.5% identical at the amino acid level over all coding
regions (Weston et al., 2002). Salmon pancreatic disease virus and SDV are very closely related to one another, but sequence comparisons and molecular phylogenetics show that they are more distantly related to other alphavirus species (30–40% amino acid identity) (Powers et al., 2001; Weston et al., 2002; McLoughlin & Graham, 2007). Nucleotide substitution rates across alphavirus genomes are nonuniform, making it difficult to estimate time of origin (Powers et al., 2001). However, alphavirus are thought to be much older than early estimates that placed their origin at several thousand years ago (Weaver et al., 1993; Weaver, 1995; Powers et al., 2001).

Pancreas disease (PD) outbreaks result in mortalities of up to 50%, causing significant economic losses for the salmonid aquaculture industry in Europe. Clinical signs of PD include lesions in the pancreas, skeletal muscle, and heart (Munro et al., 1984; Ferguson et al., 1986). SAV1 was first isolated in 1995 by serial passage of infected Atlantic salmon kidney cells with Chinook salmon (Oncorhynchus tshawytscha) embryo cells (Nelson et al., 1995; McLoughlin & Graham, 2007). Diagnostics for PD use clinical signs, histopathology, virus isolation, and RT-PCR assays with primers designed from viral genes (McLoughlin & Graham, 2007 and references therein). Quantitative RT-PCR (QPCR)-based methods developed to quantify SAV in host tissues will likely improve molecular diagnostics, aid in future studies of pathogenesis, and allow rapid screening of broodstock to identify asymptomatic carriers of SAV (Hodneland et al., 2005; Graham et al., 2006; Andersen et al., 2007).

An alphavirus (southern elephant seal virus, SES) has been isolated from a species of blood-sucking louse (Lepidopterus macorhini) that parasitizes southern elephant seals (Mirounga leonina) (La Linn et al., 2001). Although a disease in elephant seals has not been directly documented to be associated with SES infection, serological testing of seals showed that most seals older than 2 years carried antibodies that neutralized SES (La Linn et al., 2001).

**Roniviridae**

The family Roniviridae includes yellow head virus (YHV), the causative agent of yellow head disease, which infects a wide variety of species of shrimp, prawns, and krill. This disease emerged in 1990 in Thailand, and causes massive losses in the global shrimp aquaculture industry (Tirasophon et al., 2005; Intorosoot et al., 2007 and references therein). YHV is an enveloped, rod-shaped particle c. 40 nm by 170 nm with a positive-sense, c. 26-kb ssRNA genome (Assavalapsakul et al., 2006 and references therein; Intorosoot et al., 2007). Four ORFs encoding structural and nonstructural proteins have been identified in the YHV genome; the functions of some of the encoded putative proteins are not yet known (Sittidilokratna et al., 2002; Sripahijit et al., 2007). Clinical signs of infection include yellowing of the cephalothorax and gills, and erratic swimming behavior (Chantanachook et al., 1993; Intorosoot et al., 2007). Although effective vaccines or therapeutics against YHV are not yet available, recent work on the molecular pathogenesis of yellow head disease may lead to novel strategies to combat the virus. For example, in vitro and in vivo applications of RNA interference (RNAi) (Lu & Sun, 2005) with dsRNA corresponding to YHV genes have successfully inhibited viral replication (Tirasophon et al., 2005, 2007). This work may lead to the development of new RNAi-based methods for combating yellow head disease and other viral infections that threaten the shrimp aquaculture industry (Tirasophon et al., 2005, 2007). Other recent research has focused on identifying and validating shrimp genes and proteins involved in host–virus interaction and host response to the virus (Rattanarojpong et al., 2007; Sripahijit et al., 2007). Elucidating the molecular mechanisms involved in host–virus adhesion, virus uptake into host cells, virus replication within host cells, and host-defense response to the virus, may contribute to the development of new strategies for preventing, curing, and mitigating damage caused by YHV infection through vaccines, RNAi-based treatments, or drugs targeted to molecular pathways altered in the host following contact with the virus.

**Flaviviridae**

Viruses in the family Flaviviridae have a genome consisting of one monocistronic c. 11-kb RNA molecule, are known to infect a diverse collection of vertebrates, and are usually transmitted by arthropod vectors (Thiel et al., 2005). Flaviviruses, such as Tyuleni virus (TYUV), have been found in seabirds of the Alcidae family [tufted puffin (Fratercula cirrhata), common murre (Uria aalge), thick-billed murre (U. lomvia); reviewed in Muzaffar & Jones (2004)]. Similar to viruses in the families Bunyaviridae and Reoviridae, transmission of these viruses in seabirds is apparently through terrestrial invertebrate vectors such as ticks (reviewed in Muzaffar & Jones, 2004).

**Unclassified viruses**

Prokaryotic hosts

There is currently only one reported marine RNA phage. This is a 60 nm enveloped icosahedral ssRNA virus, 06N-58P, isolated from coastal Japanese waters infecting a marine strain of Pseudomonas (Hidaka, 1971; Hidaka & Ichida, 1976). Unfortunately, nothing is known about the taxonomy of this phage. There are two families of phages with RNA genomes that infect Bacteria, the Cystoviridae and the Leviviridae, neither of which are yet known to include
viruses with marine hosts (Bollback & Huelsenbeck, 2001; Mertens, 2004). There are currently no viral isolates from marine members of the Domain Archaea, but all other archaecal phage isolates known to date have linear or circular dsDNA genomes (Prangishvili et al., 2006a, b).

**Protistan hosts**

The most significant advances in understanding the virology of any of the known protist-infecting RNA viruses have been with the virus HcRNAV that infects the dinoflagellate *Heterocapsa circularisquama* (Tomaru et al., 2004b). Dinoflagellates are a diverse group of protists that include autotrophs, mixotrophs, osmotrophs (capable of the direct uptake of dissolved organic compounds), symbionts, and parasites (Graham & Wilcox, 2000). *Heterocapsa circularisquama* is a toxic bloom-forming alga responsible for mass mortality of shellfish in Japanese waters (Matsuyama et al., 1999) and there are multiple viruses known that infect *H. circularisquama* (Nagasaki et al., 2004a, 2006; Tomaru et al., 2004b, 2007). One study (Nagasaki et al., 2004a) demonstrated that during the peak of a *H. circularisquama* bloom, a remarkable 88% of cells contained virus-like particles resembling HcRNAV. Moreover, a clear correlation of HcRNAV abundance in the water and sediments with the dynamics. Another interesting finding from this system with potentially important ecological implications comes from a recent paper that showed the host dinoflagellate could display distinct responses to infection by HcRNAV. Some *H. circularisquama* strains display a ‘delayed-lysis’ property where they appear resistant to infection and no culture lysis is detected, but these cultures actually produce as much progeny virus as is made in a completely lysed culture (Mizumoto et al., 2008).

HcRNAV particles are nonenveloped, c. 30 nm in diameter, and contain a positive-sense ssRNA genome (Nagasaki et al., 2004a, 2005; Tomaru et al., 2004b). The HcRNAV genome is 4.4 kb, lacks a poly(A) tail and contains two ORFs (Fig. 4). ORF 1 has identifiable serine protease and RdRp motifs, and ORF 2 encodes a single structural protein (Nagasaki et al., 2005) 38 kDa in size (Tomaru et al., 2004b). The pathology of HcRNAV infection is very similar to what is seen with HaRNAV and *H. akashiwo*; cells infected with HcRNAV show a vacuolated cytoplasm, with fibrils in the vacuoles, and large numbers of particles accumulating that can form crystalline arrays (Nagasaki et al., 2004a; Tomaru et al., 2004b). One estimate of viral burst size was c. 20 000 viruses per cell (Tomaru et al., 2004b). Phylogenetic analyses based on RdRp alignments suggest HcRNAV is related to viruses from the *Luteoviridae*, *Barnaviridae* and *Tetraviridae* that infect plants, fungi, and insects, respectively (Fauquet et al., 2005), but falls outside these families (Nagasaki et al., 2005) and likely represents a novel family. Interestingly, a virus has subsequently been discovered and partially characterized that infects shrimp that also shows a relationship to these same families (Sritunyalucksana et al., 2006).

Many strains of HcRNAV have been isolated and these strains divide into two distinct ecotypes (UA and CY) that have distinct host ranges (Tomaru et al., 2004b). These ecotypes have almost completely mutually exclusive host ranges (based on c. 6000 cross-infection combinations tested), and it appears these virus ecotypes are important for regulating the strain diversity of the host dinoflagellate and the total biomass of the host (Nagasaki et al., 2004a, 2006; Tomaru et al., 2007). The differences between the viral ecotypes result from specific differences in the genome sequences, which are c. 97% identical (Nagasaki et al., 2005). In the viral ORF-1, which is predicted to encode the nonstructural proteins of the virus such as the RdRp, one ecotype (UA) has a 15 nt deletion relative to the other (CY). There is also a difference in the 3′-UTR regions of the sequenced strains. The relevance of these differences to the host-strain properties of the viruses is currently unknown. The other differences between the ecotypes are in the capsid protein-encoding ORF-2. There are 14 amino acid substitutions between the sequenced viral strains from each ecotype that cluster into four regions, and these regions of difference are preserved in all other viral strains from each ecotype that have been characterized. Modeling of the potential three-dimensional structures of the capsid proteins suggests that the residues where substitutions occur are all located on the exterior surface of the virus. Thus these differences likely affect host-recognition and/or entry and/or uncoating and explain the exhibited differences in host-strain specificity. This explanation is supported by further experiments in which the genomic RNAs from the two ecotypes were transected into cells that cannot be infected by the intact particles and viral replication ensued (Mizumoto et al., 2007). This seems to be an ongoing example of evolutionary ‘cat and mouse’ between the host and virus, with viral host-range variants arising through selection on mutants produced during genome replication by the error-prone RdRp enzyme (Domingo et al., 1996; Jenkins et al., 2002; Holmes, 2003; Moya et al., 2004). The research groups studying HcRNAV are making great advances to understanding the biology of this novel model RNA virus–host system and it will be interesting to track future developments.

**Invertebrate hosts**

A new RNA virus with unresolved taxonomic affiliations was recently identified in black tiger shrimp (*Penaeus monodon*) (Sritunyalucksana et al., 2006). Standard molecular
techniques (random-primed RT-PCR with shrimp hemolymph nucleic acid template, random cloning, and DNA sequencing) resulted in the identification of a cDNA with similarity at the amino acid level to RdRp sequences from viruses in the family Luteoviridae and a mushroom bacilliform virus (Sritunyalucksana et al., 2006). Based on a phylogenetic tree arising from alignments of RdRp deduced amino acid sequences, mushroom bacilliform virus and the newly identified shrimp virus (named Laem–Singh virus because it was discovered in the Laem–Singh district of Thailand) were distinct from clades containing representatives of virus families Luteoviridae, To gasviridae, and Flaviviridae (Sritunyalucksana et al., 2006). Additional sequence information from this novel RNA virus may improve our understanding of its phylogenetic relationships (Sritunyalucksana et al., 2006). Furthermore, it will be interesting to learn if there is any relationship of this virus to the dinoflagellate-infesting virus HcRNAV, which also appears to be most closely related to (but outside of) the Luteoviridae, Barnaviridae and Tetraviridae (Nagasaki et al., 2005).

### Negative-sense ssRNA viruses

Viruses from four of the 11 accepted families of negative-sense ssRNA viruses (Fauquet et al., 2005) are known to infect marine hosts: Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, and Bunyaviridae. These include some of the most devastating viruses for commercial aquaculture, and also viruses capable of transmission to humans.

#### Orthomyxoviridae

Members of the family Orthomyxoviridae, including the influenza viruses and infectious salmon anemia virus [ISAV, the causative agent of infectious salmon anemia (ISA)], have segmented, negative-sense, ssRNA genomes (Mjaaland et al., 1997). Orthomyxoviruses have genomes with six to eight segments, with segments ranging in size from c. 0.9 to 2.4 kb and total genome sizes ranging from 10 to 14.5 kb (Mjaaland et al., 1997 and references therein). Outbreaks of ISA have been reported in farmed Atlantic salmon, and asymptomatic infections have been reported in wild Atlantic salmon and other fish species including sea trout (Salmo trutta), Arctic char (Salvelinus alpinus), and herring (Clupea harengus) (Kibenge et al., 2004 and references therein). Signs of this disease include pale gills, severe anemia, enlargement of the liver and spleen, and liver necrosis. ISA outbreaks have resulted in significant losses of marine farmed Atlantic salmon in countries including Canada, US, Norway, and Scotland (Snow et al., 2003). DNA sequence differences between European and Canadian ISAV isolates show that the virus is capable of rapid evolution (Mjaaland et al., 2002), a characteristic ISAV shares with influenza viruses.

Functional genomic techniques are now being used to study fish responses to important viral pathogens such as ISAV. A recent study used an 1800-gene (1.8 K) salmonid cDNA microarray platform to study Atlantic salmon tissue (gill, heart, liver, spleen) gene expression responses to ISAV (Jorgensen et al., 2008).

Within the Orthomyxoviridae, ISAV and influenza A viruses can be considered marine RNA viruses because ISAV infects salmonids in sea water (Nylund et al., 2003) and influenza A viruses infect marine mammals and birds. Although ISAV and influenza viruses share some characteristics, such as genome size, number of genome segments, and the presence of genes encoding nucleoprotein (NP) and the three polymerase protein subunits (PB1, PB2, and PA) that make up the heterotrimeric RdRp, comparisons of their PB1 amino acid sequences show that ISAV and other orthomyxoviruses are only distantly related and ISAV may represent a new genus within this family (Krossoy et al., 1999).

Influenza viruses have been the subjects of much research and attention for a number of years, and especially in the past 3 years with an outbreak of a highly pathogenic strain in wild birds in Asia (Chen et al., 2005; Liu et al., 2005). Influenza viruses are known from a number of marine bird species (reviewed in Olsen et al., 2006), including species that spend all of their time on the ocean and only come to land for breeding. Influenza viruses are shed in the feces of infected birds (Webster et al., 1992), and can show a high incidence of infection within a population (e.g. Runstadler et al., 2007), and so waters near breeding colonies could have high concentrations of these viruses. This is especially true for some species of marine birds that can breed in very large colonies numbering in the millions of individuals. Similarly, for some species of shorebirds which also carry influenza viruses (Makarova et al., 1999; Widjaja et al., 2004; Olsen et al., 2006), flocks containing many thousands of birds can stop at specific tidal flats or similar areas during migration and so there could be high concentrations of virus particles in some of these locations at some times. Influenza viruses have been detected in nonmarine environmental reservoirs including lake water (Ito et al., 1995) and pond sediments (Lang et al., 2008) in Alaska, and lake ice in Siberia (Zhang et al., 2006), and so it may just be a matter of time before they are reported from similar samples in the marine environment. This will certainly bear more research as people try to understand the ecology of influenza viruses in natural populations and how this relates to the transmission to humans.

Birds are believed to be the primary hosts for influenza viruses, but they are also found in marine mammals (Webster et al., 1992; Van Bressem et al., 1999). An outbreak on the northeast coast of the United States killed 20% of the region’s harbour seals (Phoca vitulina) (Geraci et al., 1982),
and the most plausible explanation is that the virus originated in birds (Webster et al., 1992). Another outbreak in 1992 in the same area was caused by a distinct virus subtype, but was again believed to have originated from birds (Callan et al., 1995). Similarly, two strains of influenza isolated from pilot whales (Globicephala melaena) were very closely related to viruses from birds (Hinshaw et al., 1986). Serological studies suggest infection of marine mammals by influenza viruses could be very widespread (Nielsen et al., 2001; Ohishi et al., 2006 and references therein). Like the caliciviruses discussed previously, influenza viruses appear to regularly move between host species, including in the marine environment.

**Paramyxoviridae**

In the family Paramyxoviridae, phocine distemper virus (PDV) is a negative-sense ssRNA morbillivirus that has decimated European seal populations (Barrett et al., 2003). Walruses have also tested positive serologically for PDV (Nielsen et al., 2000; Philippa et al., 2004). Cetacean morbillivirus (CeMV) and its variants have been isolated from numerous species of whales, dolphins and porpoises, and outbreaks can have significant effects on marine mammal populations (Van Bressem et al., 1999). CeMV pathology is frequently present in stranded animals (Taubenberger et al., 2000).

There has also been a report of Newcastle disease virus in the common murre (Uria aalge), a widely distributed seabird of the northern hemisphere (Muzaffar & Jones, 2004 and references therein).

**Rhabdoviridae**

Viruses within the Rhabdoviridae family have a broad host range (e.g. mammals, fish, and plants), and include important pathogens of wild and cultured fish in both marine and freshwater environments (Hoffmann et al., 2005). The rhabdovirus genus *Novirhabdovirus* includes well-known finfish pathogens such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), Hirame rhabdovirus (HIRRV), and snakehead rhabdovirus (SHRV). The genus *Vesiculovirus* includes primarily viruses that infect fish, such as spring viremia of carp virus (SVCV) that infects and causes disease in both fish and penaeid shrimp (Lu et al., 1991; Hoffmann et al., 2005).

The 11–12 kb, nonsegmented, negative-sense, ssRNA genomes of fish rhabdoviruses contain a classical set of five genes in the following order from 3’ to 5’: N (nucleocapsid protein gene), P (phosphoprotein), M (matrix protein), G (glycoprotein), and L (RdRp) (Hoffmann et al., 2005; Kim et al., 2005). In addition to this set, viruses in the genus *Novirhabdovirus* have a gene encoding a nonvion (NV) protein of unknown function located between G and L (Fig. 6) (Hoffmann et al., 2005).

The causative agent of viral hemorrhagic septicemia (VHS) is referred to as either VHSV or Egvted virus. VHSV infections, which manifest as hemorrhaging in various tissues, have been a particular problem for rainbow trout aquaculture in Western Europe (Einer-Jensen et al., 2004; Skall et al., 2005). VHS was first described as a rainbow trout disease in 1938, and its viral etiology was confirmed in 1963 (Jensen, 1963; Skall et al., 2005). The virus was first isolated from wild marine fish (Atlantic cod) in 1979 (Jensen et al., 1979; Skall et al., 2005). In the last 20 years, VHSV has been isolated from several species of wild caught fish (e.g. Coho salmon (Oncorhynchus kisutch), Pacific cod (Gadus macrocephalus), Pacific herring (Clupea pallasi), Greenland halibut (Reinhardtius hippoglossoides), and Japanese flounder (Paralichthys olivaceus)) from marine environments in Europe, North America, and Asia (Skall et al., 2005). VHSV has been isolated from fish (e.g. Pacific herring) collected following mass mortality events off the coasts of Alaska and British Columbia (Meyers, 1999; Skall et al., 2005). In Pacific herring, VHSV appears to be triggered by stressors such as exposure to toxicants, coinfections, and nutritional stress (Meyers et al., 1994; Meyers & Winton, 1995; Skall et al., 2005). VHSV may then be transferred from Pacific herring to other fish (e.g. salmonids) via predation (Skall et al., 2005).

Molecular phylogenetic analysis based on alignment of the entire glycoprotein (G) gene nucleotide sequence of VHSV, and involving virus isolates from freshwater rainbow trout and various wild marine fish (e.g. cod, herring, and turbot), showed that all the freshwater isolates were of a single genogroup (genogroup I) while the marine isolates fell into all four known VHSV genogroups (I, II, III, and IV) (Einer-Jensen et al., 2004). VHSV in the farmed rainbow trout likely arose from VHSV in a marine environment. Wild marine finfish may represent a reservoir of VHSV and a threat to susceptible coastal fish, and also may themselves be susceptible to disease outbreaks (Einer-Jensen et al., 2004; Mork et al., 2004). Comparisons of G gene nucleotide substitution rates among freshwater or marine VHSV isolates showed that the freshwater virus evolved with a nucleotide substitution rate c. 2.5 times higher than the marine virus (Einer-Jensen et al., 2004). Many factors, such as higher temperature causing higher viral replication rate and higher density and stress in farmed rainbow trout altering response to pathogens, could influence the rate at which VHSV evolves and help to explain the apparently accelerated evolution of the virus in the cultured freshwater fish (Einer-Jensen et al., 2004).

A recent survey of wild marine fish caught in Puget Sound (Washington, DC) identified a novel rhabdovirus in a healthy starry flounder (*Platichthys stellatus*) (Mork et al.,
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Mouriyan virus (MoV), a pathogen of penaeid shrimp (Sellars et al., 2006), is morphologically similar to bunyaviruses (e.g. enveloped, spherical/ovoid, 85–100 nm diameter), and genomic sequence analysis suggests that MoV is related to viruses in the genus Phlebovirus within family Bunyaviridae (Rajendran et al., 2006 and references therein). Bunyavirus genomes have three segments of negative-sense RNA: L (large), encoding polymerase; M (medium), encoding two glycoproteins and a nonstructural protein; S (small), encoding nucleocapsid and nonstructural proteins (Soldan & Gonzalez-Scarano, 2005 and references therein). A recently developed QPCR assay for determining MoV load in shrimp tissues will be a valuable tool for producing pathogen-free broodstock (Rajendran et al., 2006).

A number of bunyaviruses from the genera Nairovirus and Phlebovirus (e.g. Avalon, Farallon, and Zaţiv Terpeniya) have also been found in seabirds of the Alcidae family (reviewed in Muzaffar & Jones, 2004). Similar to viruses in the families Flaviviridae and Reoviridae, transmission of these viruses in these seabirds is apparently through terrestrial invertebrate vectors such as ticks (reviewed in Muzaffar & Jones, 2004).

**dsRNA viruses**

There are eight families of dsRNA viruses recognized by the International Committee for the Taxonomy of Viruses (Mertens, 2004) and at least two of these families (Bunyaviridae and Reoviridae) include viruses that are pathogenic to marine organisms. There is another virus that infects shrimp that may belong in the family Totiviridae, and retroviral sequences have been amplified using PCR from marine fish.

**Reoviridae**

Viruses in family Reoviridae have segmented genomes (10–12 segments) and icosahedral capsids 70–90 nm in diameter (Mertens, 2004). Many of the reoviruses that have been isolated from marine organisms fall within the genus Aquareovirus (Samal et al., 2005), although this genus also includes viruses that infect nonmarine species. Aquareoviruses infecting a diverse range of marine species are known, including 13p2 from American oyster (Crassostrea virginica) (Meyers, 1979), CSRV from chum salmon (Oncorhynchus keta) (Winton et al., 1987), and a number of others that infect other fish (Attoi et al., 2002). Three other marine animal reoviruses, all infecting crabs, have been isolated: P from Mediterranean swimming crab (Macropus depurator) (Montanie et al., 1993), W2 from Mediterranean shore crab (Carcinus mediterraneus) (Mari & Bonami, 1988), and mud crab reovirus (MCRV) from mud crab (Scylla serrata) (Weng et al., 2007). The MCRV virus causes a ‘sleeping disease’ in infected animals and has caused large losses to the crab farming industry in China. The three marine crab-infecting viruses have similar capsid morphology and cause diseases with similar clinical signs including necrosis of gills and intestines in infected hosts (Montanie et al., 1993; Weng et al., 2007). However, W2 and P have genomes comprised of 12 RNA segments (Montanie et al., 1993), while MCRV has 13 RNA segments (Weng et al., 2007) making it the first reovirus with more than 12 genome segments. These three viruses remain unassigned to any genus (Mertens et al., 2005; Weng et al., 2007), and genome sequence is unfortunately not yet available for these viruses.

A reovirus infecting a marine protist has also been characterized. MpRV is a dsRNA virus that infects...
Micromonas pusilla (Brussaard et al., 2004). Micromonas pusilla is a globally distributed flagellated marine phytoplankter identified as the most abundant picocelluloyte (< 2 μm) in oceanic and coastal regions (Not et al., 2004). Addition of MpRV to M. pusilla cultures results in a noticeable decline in algal cells after 40 h, coincident with viral release, and the burst size has been estimated at c. 500 particles per cell (Brussaard et al., 2004). The strain of virus that was isolated and characterized showed specificity for a host strain of M. pusilla that originated from the same geographic area (Norway) and was not able to infect strains from other locations.

The MpRV virions are 65–95 nm in diameter and appear to have multiple protein layers (Brussaard et al., 2004; Attoui et al., 2006). There are five major structural proteins, and the genome is composed of 11 segments of dsRNA that total c. 25.5 kbp (Brussaard et al., 2004). The genome has been sequenced and phylogenetic analyses confirmed the placement of MpRV within the family Reoviridae, although it appears to represent a new genus, Mimoreovirus, based on these analyses as well as several unique genomic features (Attoui et al., 2006). Putative cell attachment, capsid and nonstructural proteins, including RdRp were identified in the genome sequence based on similarity to known viral sequences (Attoui et al., 2006). It will be interesting to learn more about the ecology and biogeography of this virus. The discovery of this protist-infecting reovirus suggests the marine environment could continue to be a source of novel reoviruses infecting diverse hosts.

A number of reoviruses, (e.g. Bauline virus, Kemerovo virus, and Okhotskiy virus) apparently all belonging to the species Great Island virus, have also been found in seabirds of the Alcidae family (reviewed in Muzaffar & Jones, 2004). Similar to viruses in the families Bunyaviridae and Flaviviridae, transmission of these viruses in seabirds is apparently through terrestrial invertebrate vectors such as ticks (reviewed in Muzaffar & Jones, 2004).

Birnaviridae

Aquatic birnaviruses have a global distribution, and are able to infect a variety of fish and shellfish species including Atlantic salmon, red sea bream (Pagrus major), American plaice (Hippoglossoides platessoides), Atlantic cod (G. mormyra), Japanese pearl oyster (Pinctada fucata), and jackknife clam (Sinonovacula constricta) (Kusuda et al., 1994; Suzuki et al., 1997, 1998; Suzuki & Nojima, 1999; Kitamura et al., 2000; Takano et al., 2001).

Members of the genus Aquabirnavirus in family Birnaviridae have nonenveloped, icosahedral capsids 60–70 nm in diameter, and genomes composed of two segments, A and B, of dsRNA (Fig. 6) (Dobos et al., 1979; Mertens, 2004; Nishizawa et al., 2005; Pedersen et al., 2007). Segment A is 3.1 kb long and contains two ORFs: one encodes a 17-kDa nonstructural protein, and the other encodes a 106-kDa polyprotein (pVP2-VP4-VP3) that is proteolytically processed by VP4 (a serine-lysine protease also called NS) to form the main structural constituents of the viral particle (Duncan & Dobos, 1986; Duncan et al., 1987; Pedersen et al., 2007). Segment B is 2.8 kb long and encodes the 94 kDa VP1, a RdRp required for viral replication and transcription (Romero-Brey et al., 2004; Nishizawa et al., 2005; Pedersen et al., 2007). Using various molecular techniques including the yeast two-hybrid system and deletion mapping, it was recently shown that IPNV VP3 interacts with itself, VP1, and dsRNA, and that the VP1–VP3 complex likely acts in virion assembly (Pedersen et al., 2007).

Phylogenetic analyses with a portion of segment A PCR amplified from 93 worldwide aquabirnavirus isolates showed that the isolates formed seven genogroups (Nishizawa et al., 2005). One of these clades (genogroup VII) was composed entirely of Japanese isolates from a variety of marine teleost hosts including Japanese flounder, rainbow smelt (Osmerus mordax), and yellowtail (Seriola quinqueradiata), while another clade (genogroup I) contained both Japanese and US isolates from freshwater salmonid hosts (Nishizawa et al., 2005). Therefore, Nishizawa et al. (2005) deduced that genogroup I of aquabirnavirus may have been introduced to Japanese freshwater environments in the 1950s when Japan was importing large amounts of fish and fish gametes from North America (Yoshimizu, 1996; Nishizawa et al., 2005). Interestingly, some genogroups contain isolates from both vertebrate and invertebrate hosts; for example, closely related aquabirnaviruses in genogroup II infect clam, eel, trout, and perch (Nishizawa et al., 2005). This work demonstrates the use of molecular methods for characterizing viral subtypes and for studying the origin and migration of marine RNA virus populations.

A well-studied member of the Aquabirnavirus is Infectious Pancreatic Necrosis Virus (IPNV), the causative agent of Infectious Pancreatic Necrosis (IPN). IPNV primarily causes disease in freshwater stages of salmonids including rainbow trout, brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), Atlantic salmon and some species of Pacific salmon (Onchorhynchus spp.). Disease and high mortality often occurs in first-feeding fry (early juveniles) in freshwater environments, and also in smolts after they move from a freshwater to a seawater environment (Reno, 1999; Larsen et al., 2004; Lockhart et al., 2004; Romero-Brey et al., 2004). Disease caused by IPNV is considered to be a serious threat to Atlantic salmon aquaculture in the European Union (Murray et al., 2003). This virus and serologically related IPNV-like viruses have also been reported from a wide variety of fish in freshwater, brackish, and marine environments as both subclinical covert and overt infections.
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(Reno, 1999; Nishizawa et al., 2005). This has lead to IPNV being called ‘the most common pathogenic microorganism in the aquatic fauna’ (Pedersen et al., 2007).

In salmonids, histopathology associated with IPN includes necrosis of cells in the pancreas, intestine, and liver (Smitt et al., 2006). Fish that are exposed to IPNV but either survive disease or do not develop clinical disease may become lifelong carriers of the virus, serving as IPNV reservoirs in populations and transmitting the virus vertically to progeny via the egg (McAllister et al., 1987; Pedersen et al., 2007).

**Retroviridae**

Retroviral sequences have been amplified using PCR from the DNA of two marine fishes, lemon shark (*Negaprion brevirostris*) (Martin et al., 1997), and puffer fish (*Fugu rubripes*) (Herniou et al., 1998). Because of the method by which these sequences were characterized, it is unknown if these retroviruses are currently ‘viable’ in these host species. The phylogenetic analyses that were performed with the sequences indicate they group independently from each other with various nonmarine and terrestrial retroviral sequences (Herniou et al., 1998).

**Unclassified viruses**

Infectious myonecrosis virus (IMNV), a pathogen of penaeid shrimp, has a genome of 7560 bp encoding a capsid protein, a putative RNA-binding protein, and a putative RdRp (Fig. 6) (Poulos et al., 2006). IMNV causes infectious myonecrosis, an emergent disease characterized by necrotized skeletal muscle in abdominal and tail regions and causing high mortalities in farmed shrimp (Poulos et al., 2006). Comparison of the IMNV putative RdRp sequence with other viral sequences reveals significant similarity to viruses in family Totiviridae. Viruses in family Totiviridae have nonsegmented genomes and small (30–40 nm diameter) icosahedral capsids (Mertens, 2004). Although particle size, genome organization, and molecular phylogenetic analyses based on RdRp sequence alignments suggest that IMNV may be a novel member of family Totiviridae that infects an arthropod host (with previously known totiviruses infecting only fungi and protozoa), some differences between IMNV and totiviruses, such as different sites of virus replication in host cells, raise the possibility that IMNV represents a new dsRNA virus family (Poulos et al., 2006).

**Transmission of marine RNA viruses to humans**

Most incidences of human infection by RNA viruses from marine sources likely result from the contamination of recreational sites and shellfish with sewage or other contaminated wastewater (e.g. Nenonen et al., 2008). Viruses from these sources are pollutants rather than marine RNA viruses and so are mostly beyond the intended scope of this review. For more information on this topic, we refer readers to a recent review covering this subject in depth (Griffin et al., 2003). There are, however, instances of genuine marine RNA viruses causing infections and disease in humans. Transmission between marine and terrestrial hosts appears to be a prevalent potential for marine caliciviruses (Smith, 2000). As mentioned above, marine caliciviruses have been transmitted to domesticated swine through contaminated fish scraps (Smith, 2000). A calicivirus in the genus *Vesivirus* that was isolated from a northern fur seal (*Callorhinus ursinus*) subsequently caused infection in a lab worker (Smith et al., 1998b). Interestingly, the disease was manifested in similar ways in both hosts, with blistering on the seal flippers and vesicular lesions on the hands and feet of the infected person. This same virus can also infect whales, shellfish, and fish, and therefore there are multiple potential routes of transmission of this virus from the marine system to humans (Smith et al., 1998a; Smith, 2000). It was subsequently shown that c. 20% of people tested were positive for antibodies against *Vesivirus*, and viral cDNA could be amplified from c. 10% of these that were tested (Smith et al., 2006). Given this calicivirus propensity for jumping between different host species, it is also possible that some cases of shellfish-acquired viral infections are not the result of contamination from land sources, but rather result from viruses that were filtered from the water by the shellfish or perhaps that replicate in the shellfish (Smith, 2000).

Influenza viruses (family *Orthomyxoviridae*) can also infect multiple species, and transmission between different marine hosts is known as discussed above. Birds represent the major host species and reservoir for these viruses, and many marine species of birds can serve as hosts (Webster et al., 1992; Olsen et al., 2006). Bird viruses are also believed to be the major source of new virus types infecting humans (Webster et al., 1992, 2006; Rambaut et al., 2008) and these new viruses can lead to global pandemics in extreme cases.

**Analyses of RNA viroplankton diversity**

Cultivation-independent analyses have been instrumental in revealing the extraordinary diversity of microbial life in the sea. Of particular value have been inventories of the small subunit rRNA (ssu rRNA) gene, which suggest that the diversity among marine protistan (Caron et al., 2004) and prokaryotic (Rappé & Giovannoni, 2003) plankton far exceeds that represented among current culture collections. The fraction of viruses that remains uncultivated is expected to be greater still (Rohwer, 2003), because cultivation of a
virus requires a viable host, viruses are often host-specific, and many different viruses may infect a single host strain. Although obtaining a virus in pure culture is a desirable goal, and is essential if the virus is to be fully characterized, cultivation is an impractical approach to describing diversity in natural viral assemblages (Steward & Azam, 2000). A number of alternative, cultivation-independent methods have thus been adopted which include detailed morphological descriptions (Frank & Moebus, 1987), genome size distributions (Steward et al., 2000), amplification, and sequencing of specific genes (Chen et al., 1996; Fuller et al., 1998), or sequencing of environmental shotgun clone libraries (Breitbart et al., 2002, 2004; Angly et al., 2006; Bench et al., 2007).

The first two of the above-mentioned methods have not been applied specifically to the analysis of marine RNA viruses. The major limitation of the first method is that cataloguing morphological diversity in natural viral communities by electron microscopy is laborious and provides little resolution among viruses. Because RNA-containing viruses tend to be small and untailed, there is little morphological detail that will distinguish among them or even that will conclusively distinguish them from small, untailed DNA-containing viruses. The second method is more tractable as histograms of viral genome sizes within a sample are relatively quick and easy to obtain and can provide quantitative data on diversity. Although this method has so far only been applied to DNA virus assemblages, it could be readily adapted to the analysis of RNA virus assemblages by substituting denaturing agarose gel electrophoresis for the pulsed field gel electrophoresis (PFGE) that is currently used to resolve the larger DNA genomes. Similarly, standard agarose gels or PFGE could be used to detect dsRNA viral genomes. The resolution of this method is rather limited, however, because only significant differences in genome size are discriminated, and not differences in sequence. The presence of segmented genomes could also complicate interpretation of banding patterns.

The superior resolution achievable by sequence analysis has made sequence-based methods the most popular approach to cataloguing microbial diversity and, so far, the only methods that have been applied to analysis of the RNA-containing viruses within the virioplankton. In the following sections, we discuss the advantages and disadvantages of these methods and summarize what has been discovered about marine RNA viruses by applying these techniques.

**Single-gene surveys**

In contrast to all cellular life forms, viruses have no universal gene or signature sequence that can be compared across all groups. As a result, the use of gene sequence comparisons to investigate the diversity within marine viral communities has required the targeting of different genes for different virus types. The DNA polymerase gene, for example, has been used to investigate the diversity among members of the family *Phycodnaviridae* (Chen et al., 1996), and the gene gp20 has been used to investigate cyanophages within the family *Myoviridae* (Fuller et al., 1998).

One functional gene that is present in all RNA-containing viruses, with the exception of retroviruses, is the RdRp. This gene is, therefore, an attractive marker for exploring the diversity of RNA viruses. Even though the gene is present in most RNA-containing viruses, there is insufficient nucleotide sequence conservation to allow the design of a ‘universal’ RdRp PCR assay. Consequently, assays to amplify this gene have required the design of primers that target specific subgroups.

The first successful application of an RT-PCR assay for RdRp genes in seawater (Culley et al., 2003) targeted viruses belonging to the viral order *Picornavirales* (Le Gall et al., 2008), which is a group of positive-sense ssRNA viruses with some similar genome features and sharing conserved regions in RdRp sequences. These viruses are of economic and public health concern and are responsible for animal diseases (Pallansch & Roos, 2001), plant diseases (Lazarowitz, 2001), and insect diseases (Bailey et al., 1964). Phylogenetic analyses showed that none of the environmental sequences detected fell within previously established virus families. New RdRp primer sets were subsequently designed (Culley & Steward, 2007) and used to recover additional novel RdRp sequences from several distinct aquatic environments, including an estuarine urban canal and a subtropical bay in Hawaii, and a temperate bay in California. Amplification occurred in samples from the same site in different seasons and at different depths showing that RNA viruses are widespread and consistently present. Sequences of the amplified RdRp gene fragments obtained in this second study were highly divergent from those of known viral isolates (Fig. 7), but clustered with other RdRp sequences reported previously from coastal British Columbia (Canada). The one sequence type that was recovered from the temperate California waters (Culley & Steward, 2007) was very similar (97% amino acid identity) to a sequence reported previously from coastal British Columbia (Culley et al., 2003). Similarities between subtropical and temperate sequences were all much lower (<75% amino acid identity), suggesting geographical differentiation in sequence types.

Phylogenetic analyses suggest that the environmental sequences of the RdRp gene recovered to date constitute three novel virus families, sixteen new genera and forty new species within the *Picornavirales*. RdRp sequences within this proposed order appear to cluster according to the phylogenetic affiliation of the host organisms. Most of the environmental RdRp sequences cluster with or near those derived from viruses known to infect protists, and so it has
been suggested that these represent undescribed protistan viruses (Culley et al., 2006, 2007; Culley & Steward, 2007). Bayesian clade credibility values are shown for relevant nodes, based on 10^6 generations. The Bayesian scale bar indicates a distance of 0.1. Accession numbers for viral genome sequences are: ALSV, NC_003787; BBWV, NC_005289; CPMV, NC_003549; CrPV, NC_003924; DCV, NC_001834; DWV, NC_004830; ERBV, NC_003983; FMDV, NC_011450; HaRNAV, NC_001617; NIMV, ABO22887; PV, NC_002058; PYFV, NC_003628; RsRNAV, A8243297; RTSV, NC_001632; SDV, NC_003785; SssRNAV, NC_007522; VDV, NC_006494.

Metagenomics

Although single-gene surveys can reveal novel variants of known genes, they cannot capture the full range of diversity in an assemblage. Other methods, which provide a global assessment of the sequence diversity, can provide a more comprehensive view of the diversity in a community. Metagenomics (or environmental genomics) is the sequencing of shotgun clone libraries of total community DNA extracts. The great advantage of the approach is that it is possible to discover completely unexpected genes (e.g. Béja et al., 2000). Environmental shotgun clone libraries were initially developed for analysis of prokaryote communities (Vergin et al., 1998), but are now being applied to complex, natural assemblages of marine viruses (Breitbart et al., 2002, 2004; Steward et al., 2002; Angly et al., 2006). These latter studies have shown that natural assemblages of DNA viruses are extraordinarily diverse, making the reconstruction of a genome by random sequencing an improbable event except with extraordinary sequencing effort (Angly et al., 2006).

In contrast to the case for marine DNA viruses, entire RNA virus genomes have been reconstructed using metagenomic shotgun libraries. Culley et al. (2006) constructed reverse-transcribed whole-genome shotgun libraries to
characterize two marine RNA virus communities from coastal British Columbia. Most of the sequences were unrelated to known sequences. For the sequences that were related to known sequences, positive-sense ssRNA viruses that are distant relatives of known RNA viruses dominated the samples. One RNA virus library (JP) contained diverse picorna-like virus sequences, whereas the second library (SOG) was dominated by sequences related to members of the family Tombusviridae and genus Umbravirus (Culley et al., 2006). In the SOG sample, 59% of the sequence fragments that formed overlapping contiguous sections (contigs) fell into one segment. Similarly, 66% of JP sequence fragments contributed to only four contigs (Culley et al., 2006). Using a PCR-based approach to increase the sequence coverage for each dominant contig resulted in the assembly of three complete viral genomes (Fig. 8). These presumably represent the most abundant viruses in the two marine RNA virus communities sampled. Compared with the intensive sequencing required to reconstruct prokaryotic and DNA virus genomes from natural communities, whole-genome shotgun library sequencing appears to be a reasonable strategy for reconstructing genomes of uncultivated RNA viruses.

**Concluding remarks**

It has been known for quite some time that RNA viruses cause diseases in many marine animals, but it is just in the past 5 years that their importance in plankton ecology was realized. It is now clear from cultivation-independent analyses and the characterization of new isolates that the oceans harbour an extraordinary diversity of RNA viruses. Many of the new RNA viruses infecting marine protists appear to be defining novel virus families. Some of the novel marine RNA viruses have features that link them with known groups. These viruses infect basal eukaryotes, and therefore may reflect a more ancestral state of the viruses that now infect higher organisms, such as the picornaviruses that infect mammals. In particular, the dinoflagellate-infecting virus HcRNAV is proving to be a powerful novel model virus system for the dissection of virus–host interactions. The development of techniques to get unencapsidated HcRNAV RNA into the host cells paves the way for future studies with altered virus genomes and could lead to major advancements, not only for understanding the biology of the virus but also in the cell biology of the host, analogous to what has been accomplished through the study of plant and animal viruses.

RNA viruses cause some of the most devastating diseases affecting the global aquaculture and mariculture industries. Molecular biology and genomic methods are rapidly advancing the study of some of these pathogens and the molecular mechanisms involved in host–pathogen interactions in marine vertebrates and invertebrates. These studies promise to aid in the development of new diagnostics and novel approaches and models for studying the dissemination and evolution of marine RNA viruses in populations of farmed aquatic animals. Functional genomic research techniques are being used to study host–pathogen interactions and fish responses to vaccines, paving the way for the development of new vaccines and therapeutics for combating emerging pathogens and methods for selecting disease-resistant breeding stock. This work will become increasingly important, as our growing human population demands that more food be produced more efficiently from the sea. However, such studies also contribute to the basic biology of virus–host interactions and to the understanding of viral evolution. Despite the recent advances in characterizing the diversity of marine RNA viruses, the potential for discovery still seems as vast as the ocean itself. Our challenges now are to not just describe, but also begin to make sense of this grand diversity and to work RNA viruses into the larger ecology of the sea. Continued efforts to characterize marine RNA viruses are certain to improve in fundamental ways our understanding of virus transmission, emergence, ecology, and evolution.

**Acknowledgements**

We thank Stewart C. Johnson and two anonymous reviewers for helpful comments on the manuscript. We also thank Yuji
Tomaru for sharing unpublished information about the virus CafrRNA, Kenneth Hasson for assistance with permissions for the TSV figure, and Amanda Toperoff for help with the figures. Research in A.S.L.’s laboratory is supported by grants from the Natural Sciences and Engineering Research Council (NSERC), the Canada Foundation for Innovation (CFI), and the Industrial Research and Innovation Fund (IRIF) from the Government of Newfoundland and Labrador. Research in M.L.R.’s lab is supported by grants from NSERC, CFI, IRIF, the Canada Research Chairs program, and the Atlantic Cod Genomics and Broodstock Development Project through Genome Canada. A.I.C. and G.F.S. were supported in this work by grants from the United States National Science Foundation to G.F.S. (OCE 06-0026) and to the Center for Microbial Oceanography Research and Education (OCE 04-24599).

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Lippincott, Williams, and Wilkins, Philadelphia, PA.


