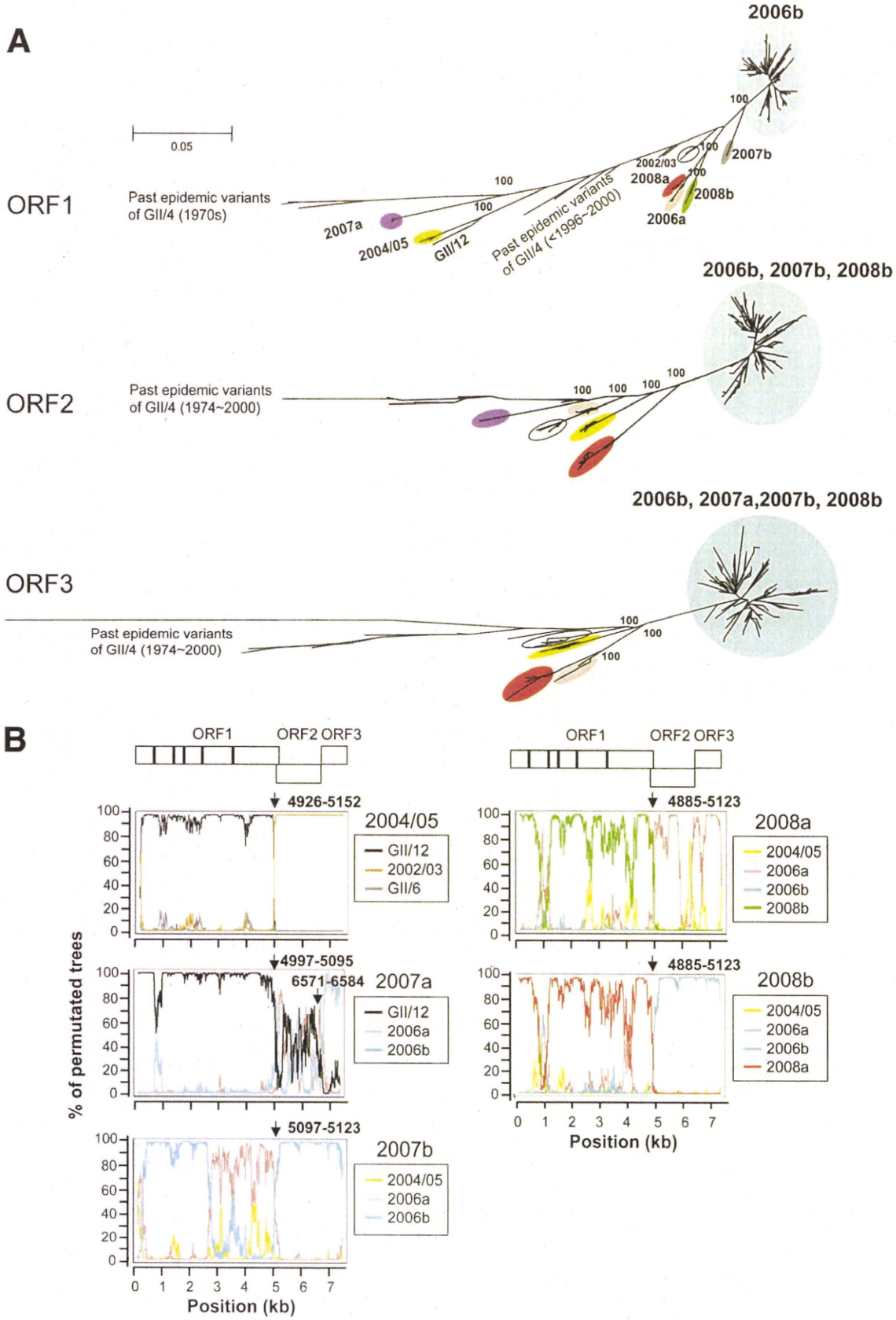

FIG. 3. Phylogenetic evidence for NoV genome mosaicism. (A) Maximum-likelihood trees of the nucleotide sequences of the complete ORF1 (about 5.1 kb), ORF2 (about 1.6 kb), and ORF3 (about 0.8 kb). The trees were constructed with the sequences obtained in previous (38) and present studies (n = 199) and the reference sequences described in Fig. 1. The GII/12 sequence (Saitama_U1/JP [25]) was used as an outgroup

ORF3 related to GII/4 2006b. The 2008a genome (Hokkaido4/2008/JP) was comprised of the ORF1 related to GII/4 2008b (Hokkaido5/2008/JP) and as-yet-undefined classes of GII/4 and the ORF2 and ORF3 of GII/4 2006a and as-yet-undefined classes of GII/4. The 2008b genome was made up of the ORF1 of 2008a and the ORF2 and ORF3 related to GII/4 2006b. We also investigated possible genome mosaicism for the 2006a and 2006b subtypes, but we could not identify putative ancestral sequences of ORF1, ORF2, and ORF3 that were genetically closely related to 2006a and 2006b when we used the available NoV sequences in the public database as references.

To define statistically the possible recombination breakpoints of the 2004/05, 2007a, 2007b, 2008a, and 2008b genomes, we performed informative-site analysis (50) using the same reference sequences used in the bootscanning-plot analysis. With this approach, we identified several patches of genome regions that were assigned with statistical significance as putative recombination breakpoints. Notably, a putative breakpoint located around the junction of ORF1 and ORF2 constantly gave the highest statistical significance, i.e., maximum χ^2 values, in the 2004/05, 2007a, 2007b, 2008a, and 2008b genomes ($P \leq 0.000001$) (Fig. 3B, arrows). The results were in good agreement with the phylogenetic-tree and bootscanning-plot analyses. These data consistently suggest that the new GII/4 subtypes identified in Japan were mostly hybrid viruses composed of viral protein elements from distinct genetic lineages of NoVs.

We further assessed possible genome recombination events using other tools included in the RDP3 software package (30). The analysis again identified single recombination breakpoints with the best or second-best confidence values around the junction of ORF1 and ORF2 in the 2004/05, 2007a, 2007b, 2008a, and 2008b genomes ($P < 0.001$). The analysis also identified additional putative breakpoints around the junction of ORF2 and ORF3 of 2007a. However, we could not obtain evidence for genome mosaicism with 2006a and 2006b using a selected sequence data set of the NoV GII genotypes reported to date (GII/1 to GII/19) (25, 65, 71) and GII/4 subtypes (7, 38, 53). Because information on the entire genome sequences of NoV is very limited, it remains to be determined whether 2006a and 2006b also have mosaic genomes.

Isolation of NoV mosaic genome segments. To clarify the presence of the mosaic viral genomes in nature, we cloned and sequenced the genomes of the 2007a, 2007b, 2008a, and 2008b subtypes. For this study, we cloned the genome segments, i.e., the 5.2-kb, 2.8-kb, and 1.0-kb genome segments, that presumably contain a junction of putative recombination breakpoints around the ORF1/ORF2 junction (Fig. 4). The subtype 2004/5 was not included in the cloning analysis because we did not have sufficient amounts of clinical specimens for the cloning. Likewise, the subtypes 2006a and 2006b were not included because the major parental sequences are not clear in the present study. We successfully obtained the molecular clones except for the 5.2-kb fragment of 2007a. We could amplify but failed to clone the 5.2-kb fragment of 2007a. Although the precise reason for the failure is unclear at present, it might be due to the decreased cloning efficiency of the larger insert by the TA-cloning method. Because the appropriate restriction enzyme sites for the cloning were absent in the 2007a 5.2-kb fragment, we did not include this fragment in further analyses. Nucleotide sequences of the segments were used for the bootscanning-plot analysis using the same sets of reference sequences described in Fig. 3B, and the statistically significant putative recombination breakpoints were assessed by informative-site analysis.

Figure 4 shows representative results of the bootscanning-plot and informative-site analyses with the 5.2-, 1.0-, and 2.8-kb segment clones. Importantly, all 11 clones from the 2007a, 2007b, 2008a, and 2008b stool specimens had the same putative recombination breakpoints, with the highest statistical significance around the ORF1/ORF2 junction region identified with direct sequencing analyses (Fig. 4A and B, arrows). In addition, the patterns of the bootscanning plots were almost identical over the viral genomes examined between the sequences of the uncloned and cloned genome segment except for the 5′-half region of the 2007b ORF1 (Fig. 3B and 4). Although the precise reason for the discrepancy is unclear at present, it might be due to the cloning of the minor population of the 2007b quasispecies in the stool specimens. The overall good agreement of the results by the two sequencing strategies strongly suggests that the genome mosaicisms we found by analysis of the direct sequencing data were intrinsic rather than an artifact of the analysis. Taken together, these data indicate that the NoV mosaic genomes were present in the human stool specimens and that the ORF1/ORF2 junction region is the common hot spot for generating the mosaic genomes in GII/4 subtypes in nature.

Amino acid signatures of the NoV GII/4 subtypes. We then investigated sequential characteristics of the proteins of the 7 GII/4 subtypes by searching for unique amino acid signatures in viral proteins. The deduced amino acid sequences of ORF1, ORF2, and ORF3 of a given subtype were aligned with reference sequences of the past GII/4 subtypes (38) that were identified before detection of the query subtype. Amino acids specific to the query subtype were extracted and referred to as amino acid signatures of the new epidemic subtype. In the case of the 2006b subtype, we also analyzed the changes in the signatures in the capsid protein VP1 between 2006 and 2009, because information on the structure and function is more abundant for the capsid than for other viral proteins.

sequence in each tree but is shown only in the ORF1 tree. In the ORF2 and ORF3 trees, the GII/12 sequence was located far apart from the GII/4 cluster and is not shown for simplicity. (B) Bootscanning plots of nucleotide sequences of near-full-length NoV genomes. A query genome sequence (2004/05, 2007a, 2007b, 2008a, or 2008b) was aligned with three reference sequences, two sequences that were positioned relatively closely to the query sequence in the neighbor-joining trees and a sequence that was distantly related to the query sequence, using CLUSTAL W software, version 1.4 (62). The bootstrap values are plotted for a window of 300 bp moving in increments of 10 bp along the alignment using the program Simplot (48). Informative-site analyses (50) were performed using the same query and reference sequence set. Arrows indicate putative recombination breakpoints with the highest statistical significance ($P \leq 0.000001$) in the informative-site analysis.

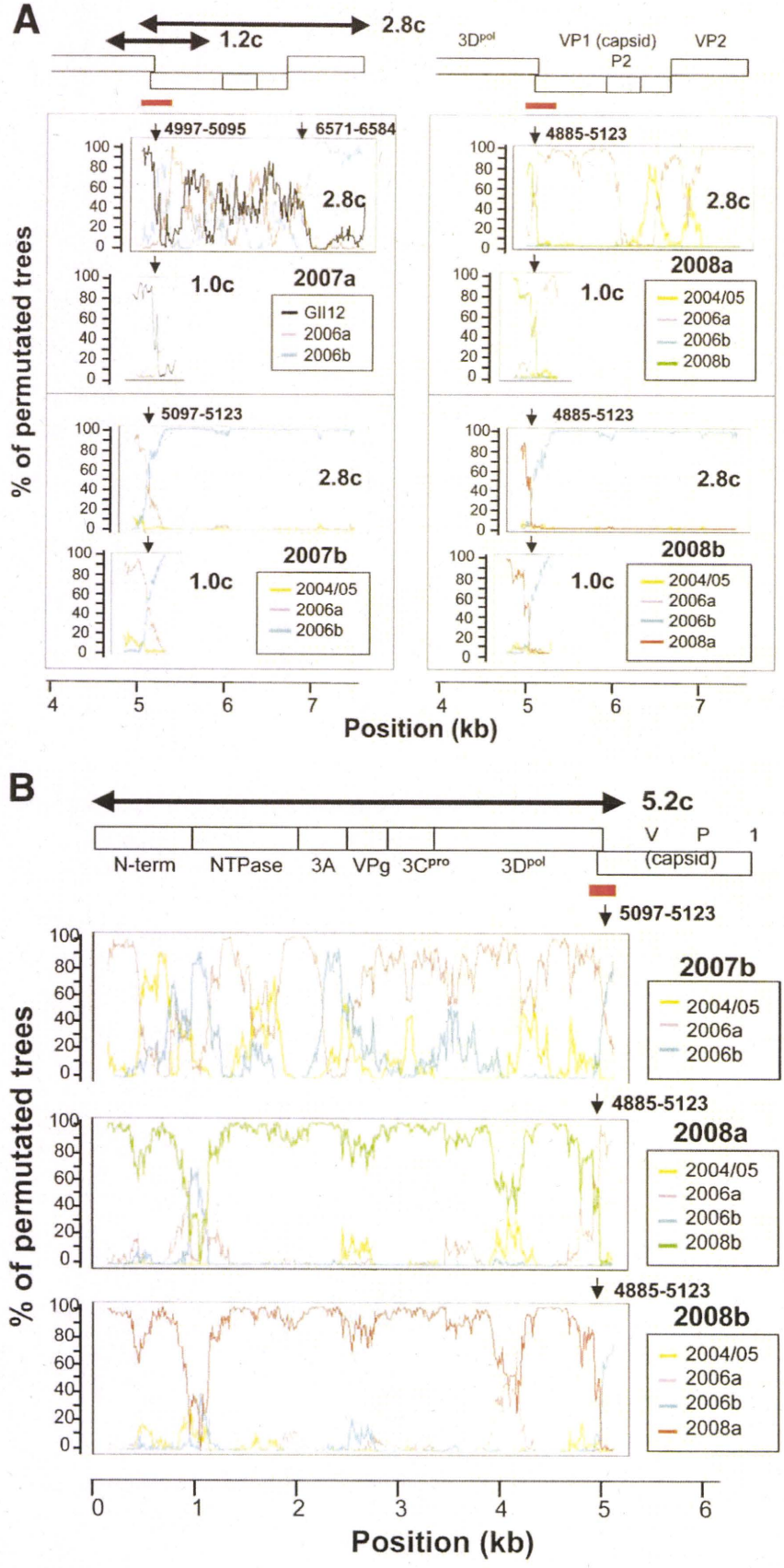

FIG. 4. Isolation of NoV mosaic genome segments. Three genome segments (5.2, 1.0, and 2.8 kb) were amplified from the 2007a, 2007b, 2008a, and 2008b stool specimens, cloned into plasmid vectors, and sequenced. Nucleotide sequences of the cloned segments were subjected to the bootscanning-plot analysis using the same sets of reference sequences described in Fig. 3B, and the putative recombination breakpoints were assessed by informative-site analysis. (A) Results for the 2.8-kb and 1.0-kb genome segment clones (2.8c and 1.0c). (B) Results for the 5.2 kb-genome segment clones (5.2c). Red bars indicate the ORF1/ORF2 bordering region. Arrows indicate the putative recombination breakpoints with the highest statistical significance ($P = 0.000001$) in the informative-site analysis.

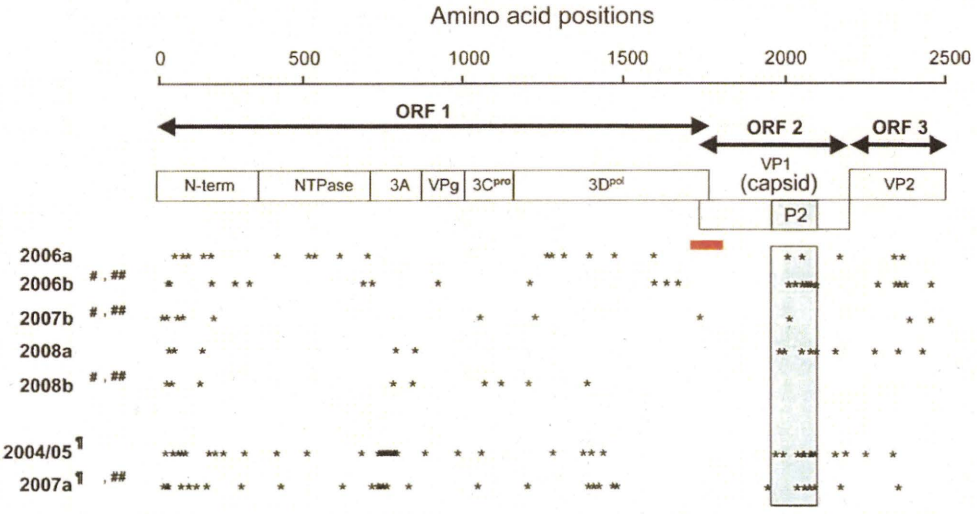

FIG. 5. Amino acid signatures of the NoV GII/4 subtypes. The deduced amino acid sequences of ORF1, ORF2, and ORF3 of a given GII/4 subtype were aligned with the GII/4 sequences identified before the outbreak season of the subtype. Amino acids specific to each subtype at the time of its first outbreak season were extracted and referred to as amino acid signatures of the new epidemic subtype. Asterisks illustrate approximate locations of the amino acid signatures in ORF1, ORF2, and ORF3. A light-blue box denotes approximate locations of the capsid P2 domain in ORF2. A red bar indicates the ORF1/2 boundary region where the single putative recombination breakpoint was assigned for each subtype genome by informative-site analyses (50). ¶, ORF1s were similar to those for GII/12 (see Fig. 4, ORF2). 2004/05 and 2007a had 27 and 63 amino acid substitutions, respectively, in ORF1s compared to the two available complete ORF1 sequences of GII/12 (accession numbers AB045603 and AB039775). #, ORF2s were classified as the same phylogenetic group (see Fig. 3A, ORF2). ##, ORF3s were classified as the same phylogenetic group (see Fig. 3A, ORF3).

The amino acid signatures of the 7 GII/4 subtypes were distributed throughout the three ORFs (Fig. 5, asterisks). 2004/5 and 2007a had more substitutions in ORF1 than the others because their ORF1s seemed to have originated with the GII/12 relatives (Fig. 3A and 4). When they were compared with the two available complete ORF1 sequences of GII/12, they still had many amino acid substitutions (27 and 63 for 2004/05 and 2007a, respectively). 2007b and 2008b had fewer substitutions in ORF2s and ORF3s than the others because these regions seemed to have originated from the 2006b relatives.

As seen in the 2006b variants in the 2006/2007 season (38), the capsid protein signatures were preferentially distributed on the P2 domain in other GII/4 subtypes (Fig. 5, blue box). All 7 capsid signatures identified in the 2006b variants in the 2006/2007 season were highly conserved during the 2006/2007 season, although two of them (P357 and N412) were gradually lost in the 2006b variant population during 2007 and 2009. Instead, other amino acid substitutions were sporadically accumulated in the P2 domain of the later 2006b variants (data not shown). The 7 signatures in the P2 domain were also well retained in the 2007b and 2008b subtypes, whose genomes had capsid gene segments from the 2006b relatives (Fig. 3B). These data indicate that (i) all of the 7 GII/4 subtypes had unique amino acid substitutions in viral capsid and replication proteins at the time of their outbreaks in Japan, (ii) the dominant 2006b subtype retained the capsid signatures during its persistence between 2006 and 2009, and (iii) some GII/4 subtypes acquired unique mutation sets of the 2006b capsid P2 domain by putative genome recombination events.

3-D locations of the subtype-specific amino acids in the capsid P domain dimer. To clarify 3-D locations of the capsid signatures, we constructed structure models of the VP1 P-domain dimer of the GII/4 subtypes by the homology modeling method as described previously (38). The 2007b and 2008b models were not included for the study because their capsid proteins had no signature or a single signature in the P2 domain due to putative genome recombination with 2006b (Fig. 3, 4, and 5). The thermodynamically and sterically optimized structural models of the P-domain dimer of the 2004/05, 2006a, 2006b, 2007a, and 2008a subtypes showed no major differences in the folding of the main chains (Fig. 6). This result suggests that the capsid amino acid substitutions primarily influenced physicochemical properties around the substitution sites by changing the size and chemical properties of the side chains. These models were then used to map the 3-D locations of the P2 domain mutations.

Importantly, the capsid P2 domain signatures were mostly mapped on the outer surface loops in all of the GII/4 subtypes examined (Fig. 6). These loops form an accessible protein surface with which host proteins, such as a cellular receptor(s) and antibodies, can directly interact. The P2 mutations are often positioned near the putative functional sites for virus entry into the cells: the fucose ring binding sites formed by the P-domain dimer (8, 13) (blue-dotted ovals) and an RGD motif (60) on the β2 sheet of the P domain (cyan chain). Notably, the P2 mutations were more abundant in the widely prevalent subtypes, 2004/05 and 2006b, than in the others (Fig. 2 and 6). The 2008a subtype, which was detected most recently and caused NoV epidemics at multiple sites in the 2008/2009 season, also had 5 unique substitutions in the P2 domain (Fig. 2 and 6). These data indicate that the GII/4 subtypes that were dominant between 2004 and 2009 in Japan had a greater number of unique amino acids preferentially positioned on their capsid surfaces at the time of their first outbreaks.

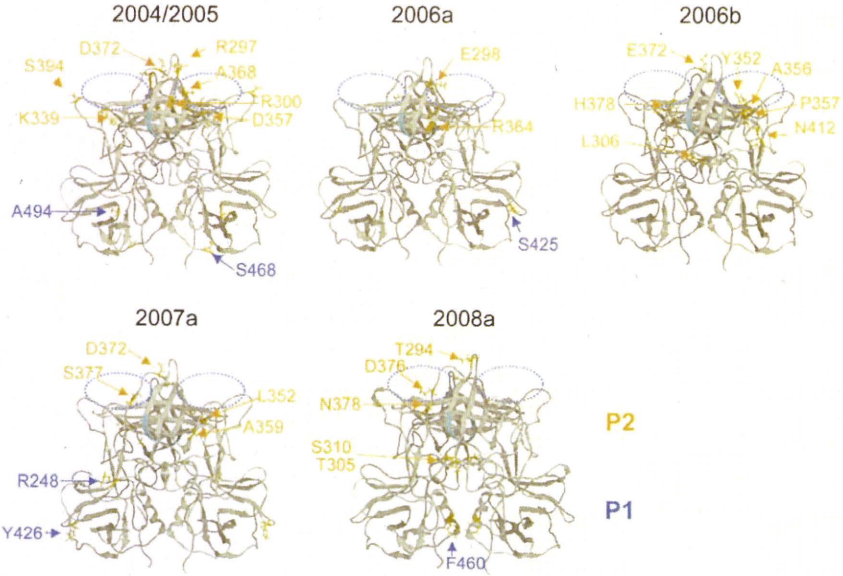

FIG. 6. 3-D locations of the subtype-specific amino acids in the capsid P domain dimer. Structural models of the capsid P domain dimers of recent NoV subtypes were constructed by homology modeling as described previously (38). The 2007b and 2008b capsid models were not included because their ORF2s were classified as belonging to the same phylogenetic group as ORF2 of 2006b due to putative genome recombination (Fig. 3 and 4), and their capsid proteins had no signature or only a single signature in the P2 domain with 2006b (Fig. 5). Orange arrows and letters indicate locations and types of the unique amino acids in each GII/4 subtype at the time of its first outbreak season. Putative functional sites for virus entry into the cells are highlighted. Blue-dotted ovals, the fucose ring binding sites formed by the P domain dimer (8, 13); cyan chain, an RGD motif (60) on the β2 sheet of the P domain.

DISCUSSION

In this study, we have examined the possible involvement of genome recombination in the generation of new outbreaks of the NoV GII/4 variants. We first analyzed the evolutionary lineage of the GII/4 variants that were present in Japan during 2006 to 2009 and clarified their temporal and geographical distribution. We showed the following: (i) that at least 7 monophyletic GII/4 subtypes were present in humans during the 3-year period, (ii) that 3 to 4 subtypes were cocirculated in each NoV season, and (iii) that the 2006b subtype had spread and persisted more effectively in human communities than the other GII/4 subtypes during the study period (Fig. 1 and 2). These and other findings on the recent GII/4 subtypes (32, 38, 53, 54) consistently support the notion that the 2006b subtype had some selective advantages over the other GII/4 subtypes, which allowed it to quickly spread throughout human communities at the time of its initial appearance in the early winter of 2006. Our study additionally suggests that the possible advantages of the 2006b subtype remained effective over the subsequent 2 years in Japan. However, we could not obtain evidence for genome mosaicism with the 2006b subtype using the available sequence data set of the NoV genogroups, genotypes, and subtypes reported to date (2, 7, 25, 38, 53, 65, 71). Therefore, it is not clear whether genome recombination played a significant role in the generation of new large-scale outbreaks. Because information on the entire genome sequences of NoV is very limited at present, further genome study of NoVs is necessary to clarify this issue.

We then analyzed the GII/4 subtypes to determine whether they showed genome mosaicisms. We demonstrated clearly and for the first time that intersubtype genome recombination is common in the new NoV GII/4 outbreaks: 4 of the 7 new GII/4 subtypes (2007a, 2007b, 2008a, and 2008b) were mosaics comprised primarily of sequences of the recently prevalent distinct GII/4 subtypes, and 1 (2004/05) was made up of GII/4 and GII/12 genotypes (Fig. 3 and 4). Because of the genome mosaicism, the number of monophyletic clusters of the new variants in the phylogenetic trees differed depending on the region of the genome studied; the numbers of clusters were 7, 7, 5, and 4 for the near-full-length genome, ORF1, ORF2, and ORF3, respectively. Sequences of 11 randomly selected genome segment clones all exhibited bootscanning-plot profiles identical to those obtained with the direct sequencing data except in one case, suggesting dominance of the specific mosaic genomes in the corresponding stool specimens. Phylogenetic-tree, bootscanning-plot, and informative-site analyses consistently provided the same conclusions in regard to genome mosaicism: these virus genomes encoded capsid proteins whose evolutionary lineages were distinct from those of nonstructural proteins. The good agreement of the results by the two sequencing strategies and by three evolutionary methods strongly suggests that the mosaic genomes made from multiple GII/4 subtypes were indeed constantly arising *in vivo* and became the dominant species in infected individuals in some of the NoV GII/4 outbreaks.

We failed to find evidence for the genome mosaicism of the 2006a and 2006b subtypes by using available NoV sequences. These subtypes are unlikely to be the intergenotype or intergenogroup recombinants, because their ORF1, ORF2, and ORF3 sequences constantly showed the strong monophyly within the GII/4 cluster out of the other genotypes and genogroups at their first (38) and successive outbreaks (Fig. 3A). However, the possibility of intersubtype recombination among as-yet-defined classes of GII/4 subtypes remains undetermined for the 2006a and 2006b subtypes.

Interestingly, the mosaic genomes that caused the new NoV GII/4 outbreaks all had the putative recombination breakpoints with the highest statistical significance in the ORF1/2 boundary region ($P \leq 0.000001$) (Fig. 3B and 4). This breakpoint location is consistent with previous reports on intergenogroup and intergenotype recombination (1, 10, 11, 17, 21, 22, 25, 40, 41, 44–46, 49, 57, 63, 64, 66), suggesting the presence of a common hot spot for generation and survival of recombinant NoVs in nature. To a lesser extent, a putative recombination event around the ORF2/3 boundary was identified in 1 of 7 new variant subgroups (Fig. 4A, 2007a). A recombination event around the ORF2/3 junction has also been reported for GII/4 variants circulating in Cairo, Egypt, between 2006 and 2007 (24). The ORF1/2 boundary region is highly conserved in NoV GII/4, as shown previously by very low scores of Shannon entropy within the reported GII/4 sequences (38). This and our present findings on the presence of the putative parent GII/4 sequences of the mosaic genomes suggest that the ORF1/2 mosaic genomes we identified were generated by homologous recombination, as seen in other single-stranded, positive-sense RNA viruses, including poliovirus (20), foot-and-mouth disease virus (36), brome mosaic virus (9, 39), turnip crinkle virus (70), and tomato ringspot virus (52). If this were the case, the intersubtype recombination at the ORF1/2 boundary region would occur and generate variable recombinant viruses *in vivo* more frequently than the intergenotype and intergenogroup recombination would, because the boundary region and neighboring sequences are more similar within the NoV subtype than within the genotype and genogroup. Our results are consistent with this possibility.

The presence of putative recombination at the ORF1/2 boundary region has a direct impact on the modes of NoV subtype evolution *in vivo*. First, the presence of the breakpoint at this region drives independent evolution of ORF1 and ORF2/3 nucleotide sequences and thus of nonstructural and capsid proteins (Fig. 3), leading to divergent evolution of the NoV GII/4 genome (Fig. 1). Second, the presence of the breakpoint allows concurrent acquisition of new mutation sets that arise independently in ORF1 and ORF2/3 among distinct GII/4 subtypes. However, further study is necessary to clarify whether the genome recombination indeed confers any fitness advantage to the virus within a mixed NoV variant population in nature.

The high levels of sequence homology of the ORF1/2 boundary region (38) also suggest that the region is functionally and/or structurally very important for NoV replication and receives strong selective constraints against diversity for NoV survival in nature. Consistently, this region is indicated to contain an important functional motif that regulates capsid expression from a full-length genome in bovine NoV (37). Thus, the ORF1/2 boundary region may be a multifunctional region critical for both replication and evolution of NoVs.

The relatively high detection frequency of the ORF1/2 mosaic genomes in the new GII/4 subtypes (5 of 7) was rather unexpected, because multiple factors, such as retention of virion stability, viral infectivity, and viral replication capabilities in human cells, should restrict the generation of viable hybrid viruses. The present findings therefore raise the possibility of large-scale coinfections by distinct lineage groups of NoVs and of natural selection for the particular ORF1/2 hybrid viruses. The former possibility remains to be clarified but is feasible (57) if one considers the high stability of the NoV virion outside the host, as well as NoV transmission modes, i.e., ingestion of contaminated food and water, direct person-to-person contact, and exposure to contaminated airborne vomitus droplets in a semiclosed community (15).

The latter possibility of natural selection also remains to be clarified. However, it is possible that some of the unique mutations identified in each ORF1/2 hybrid genome at the time of their outbreaks (Fig. 5) may be involved in the survival of the hybrid viruses. In this regard, it is noteworthy that the hybrid viruses had multiple mutations in the N-term, NTPase, 3A-like, Vpg, $3C^{pro}$, and $3D^{pol}$ proteins. These proteins are likely to function primarily in NoV replication in host cells (19). Therefore, acquisition of an appropriate mutation set in ORF1 might confer some advantages in replication of the hybrid viruses in particular hosts. It should also be noted that the 2007b and 2008b subtypes encoded the VP1 and VP2 proteins from 2006b (Fig. 3 and 4). VP1 plays critical roles in binding to the putative infection receptors (8, 60, 61) and antibody neutralization (33, 34). The VP2 protein is also essential for the production of infectious virions in caliciviruses (56). Therefore, acquisition of an appropriate mutation set in ORF2 and ORF of 2006b might confer some advantages in infection and/or immune escape of the hybrid viruses at some outbreaks.

Computer-assisted modeling studies provide a structural basis for addressing the potential selective advantages of the capsids of the new GII/4 subtypes. We showed that unique capsid amino acids of the 7 GII/4 subtypes identified in this study were preferentially positioned on the outer surface loops of the protruding P2 domain and were more abundant in the dominant subtypes (Fig. 6). This is also a common characteristic of the past epidemic GII/4 subtypes (32, 38, 53). These findings suggest that physicochemical changes in the capsid surface are a prerequisite for effective virus spread of NoV GII/4 in humans. The specific mutations around the outer surface loops of the protruding P2 domain can modulate the local electrostatic environment and shape of the exposed capsid surface by changing the chemical properties and the size of side chains, respectively. Therefore, acquisition of an adequate set of capsid P2 mutations might be able to decrease antibody affinity without decreasing affinity to the infection receptors of GII/4. This would confer an advantage to the variants that would allow them to spread in human communities in the presence of immunity against precirculated variants. To effectively gain such a set of P2 domain mutations, as well as those of nonstructural proteins, genetic recombination around the ORF1/2 boundary region may be an ideal mechanism. Establishment of a tissue culture system to support effective replication of human NoVs, as well as a reverse genetics system to study the roles of mutations in NoV infection and replication, will be critical to clarify each of these possibilities.

It should be noted that despite the prolonged dominance of the 2006b subtype, the magnitudes of NoV epidemics in Japan have gradually declined since 2007: the total numbers of reported NoV infection cases during October and March of 2007-2008 and 2008-2009 showed more than 2- and 5-fold decreases, respectively, compared with the same period in 2006-2007 under the same surveillance system (Infectious Disease Surveillance Center [http://idsc.nih.go.jp/iasr/index

.html]). These observations may imply that biological niches within human communities that support replication of the 2006b subtype have gradually been shrinking in Japan since 2007. A possible explanation for this phenomenon is that immunity against the 2006b subtype has been gradually strengthened in human populations due to the persistence of the 2006b infections in Japan. Nevertheless, none of the new GII/4 variant subtypes were able to replace the 2006b epidemic in the 2007/2008 and 2008/2009 seasons. In addition, two of the four new putative recombinants (2007b and 2008b), which appeared in the 2007/2008 and 2008/2009 seasons, gained ORF2/3 of 2006b. These observations may imply that 2006b still had some selective advantages over other GII/4 variant subgroups in the 2008/2009 seasons. Further follow-up study is necessary to address these possibilities.

Our findings on genome mosaicism may have an impact on epidemiological and virological studies of NoVs. For example, mosaicism could influence the validity of NoV classification, which is based on the sequences of parts of the NoV genome. Because hybrid viruses that cause epidemics seem to share a recombination breakpoint around the ORF1/2 boundary region, this junction segment may be useful for monitoring the prevalence of hybrid NoVs in nature. The genome mosaicism could also impact measurement of the mutation rates of NoVs in nature: careful selection of the genome segments that contain no recombination breakpoints would be critical to measure the nucleotide substitution rates. Continual accumulation of information on the complete genome sequences of NoVs in natural and living environments will provide genetic bases for dealing with these issues and illustrate mechanisms by which NoV evolves to generate and sustain new epidemics in human populations.

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NOTE

Detection of Sapovirus in oysters

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ABSTRACT

SaV sequences which are either genetically identical or similar were detected from oysters, feces from gastroenteritis patients, and domestic wastewater samples in geographically close areas. This is the first report of the detection of SaV in oysters which meet the legal requirements for raw consumption in Japan.

Key words commercial oyster, genotyping, human sapovirus, water contamination.

Outbreaks and sporadic infections of acute gastroenteritis caused by SaV have been described all over the world (1–4). *Sapovirus*, a genus of the family *Caliciviridae*, is a non-enveloped, positive-strand RNA virus (5). SaV is genetically classified into at least five genogroups, of which genogroups I, II, IV, and V infect humans (2). SaV has been detected by RT-PCR from a variety of epidemiological sources, including fecal specimens from symptomatic and asymptomatic individuals (6), environmental water (7, 8), and bivalves (9), indicating that SaV can be transmitted via fecal-oral routes through water and contaminated foods, as well as via person-to-person contact. Although several enteric viruses are associated with oyster-related gastroenteritis outbreaks (10), and SaV has been detected in feces from patients in oyster-related gastroenteritis outbreaks (11), the detection of human SaV from oysters has not been reported.

The aim of this study was to determine whether SaV could be detected in oysters for raw consumption, as well as in geographically linked settings, such as primary-treated wastewater, secondary-treated effluent from a wastewater treatment plant, oysters cultivated in an estuary receiving effluent, and fecal specimens from gastroenteritis patients. Phylogenetic analysis was performed to characterize the SaV detected in this study.

Fourteen oysters meant for raw consumption were purchased from local fish markets in Miyagi prefecture, Japan in December 2007. The other 16 oysters, immersed in river water receiving effluent from a wastewater treatment plant, were harvested after being cultivated for 2 months (October–December 2007). The oysters were shucked and digestive diverticula aseptically separated. Viruses in the digestive diverticula were eluted using a method described previously (12). Briefly, a digestive diverticulum in a polyethylene tube was mashed up with two stainless-steel beads and 1 ml of distilled, deionized water using Micro Smash MS-100 (TOMY, Tokyo, Japan) for 1 min at 4500 rpm. The supernatant was recovered by centrifugation at 10 000 rpm for 10 min and stored as a virus concentrate.

A total of 13 primary-treated wastewater samples and 13 secondary-treated effluent samples were collected from August 2007 to January 2008 from the wastewater treatment plant located near the river mouth in the oyster-harvesting area. Viruses in primary-treated wastewater and secondary-treated effluents were concentrated by the

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List of Abbreviations: GI, genogroup I; GIV, genogroup IV; SaV, Sapovirus.

polyethylene glycol precipitation method (12). In addition, SaV-positive fecal specimens from two gastroenteritis outbreaks near the investigation area during the same season (September and October 2007) were included in the analysis. Stool samples were suspended in nine times the weight of distilled, deionized water, and the supernatant collected after being centrifuged at 9200 *g* for 10 min.

Viral RNA was extracted from samples using a QIAamp Viral RNA mini kit (Qiagen, Tokyo, Japan). Complementary DNA was synthesized using a hexa-oligomer random primer with SuperScript III First-Strand Synthesis System (Invitrogen, Tokyo, Japan). The SaV gene was amplified by nested RT-PCR, F13/14 and R13/14 primers being used in the first PCR, and F22 and R2 primers in the nested PCR (13). Nested RT-PCR products were separated with 2% agarose gel electrophoresis, purified with a QIAquick Gel Extraction Kit (Qiagen), and directly sequenced by ABI PRISM 310 (Applied Biosystems, Tokyo, Japan) by using F22 and R2 primers separately. The nucleic acid sequences (approximately 320 nucleotides) were aligned, and a neighbor-joining phylogenetic tree with 1000 bootstrap replicates was generated using ClustalX software (version 2.0.10).

SaV was detected in 1 of the 14 commercial oysters (7.1%), 3 of the 16 cultivated oysters (18.8%), 8 of the 13 primary-treated wastewater samples (61.5%), and 2

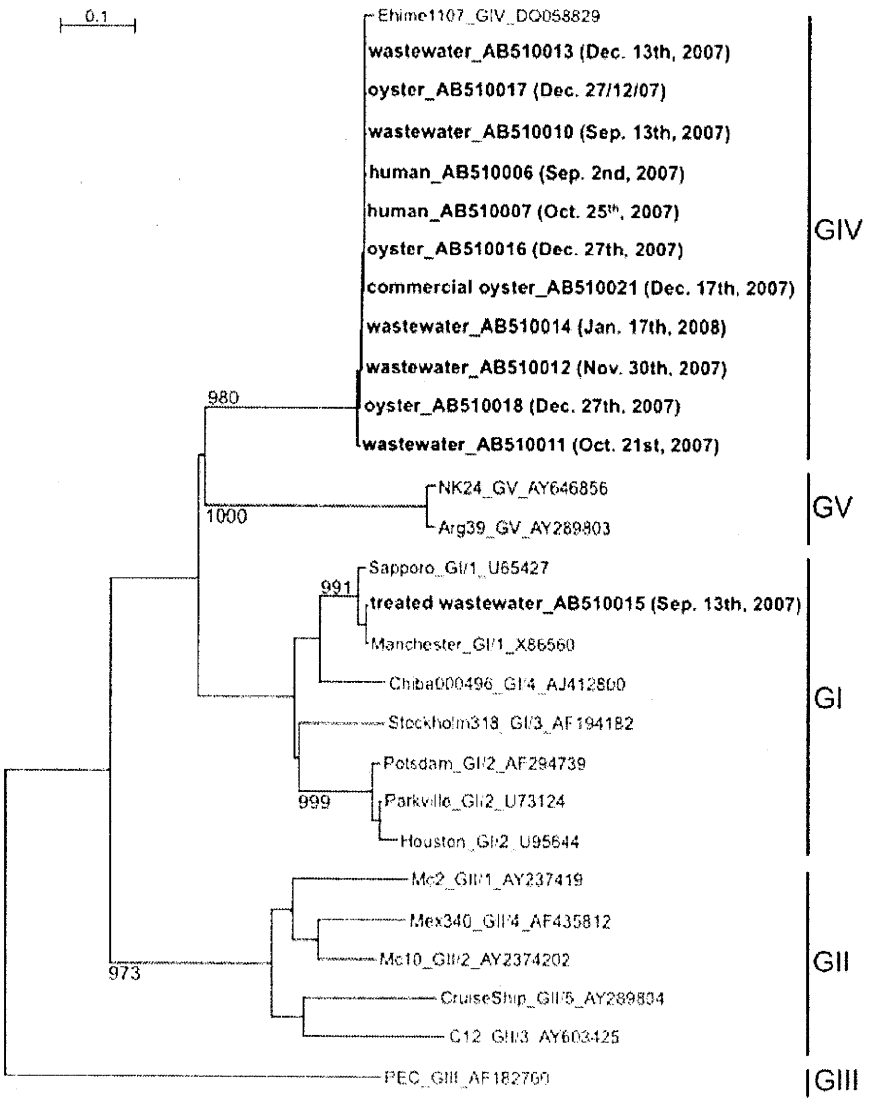

Fig. 1. Neighbor-joining phylogenetic tree of Sapovirus capsid nucleotide sequences acquired in this study. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values greater than 950 were considered statistically significant for the grouping. The scale bar represents nucleotide substitutions per site. Strains with bold characters were obtained in this study, and the date of sampling is given in the parenthesis of each strain name.

of the 13 secondary-treated effluent samples (15.4%). The nucleotide sequences of the partial capsid gene were determined from 10 samples in total (all positive oyster samples, five primary-treated wastewater samples and one secondary-treated effluent sample). Sequences in the remaining four positive samples were difficult to determine by the direct sequencing approach, probably because of the presence of mixed sequences. Phylogenetic analysis based on the determined sequences revealed that the SaV detected in the commercial oysters for raw consumption (accession number: AB510021) belonged to GIV (Fig. 1). Genetically similar or identical sequences of SaV GIV (99.4–100% nucleotide identities in 321 nucleotides) were detected from three cultivated oysters (AB510016–AB510018), five primary-treated wastewater samples (AB510010–AB510014), and two fecal specimens (AB510006 and AB510007) collected during the same season (Fig. 1). SaV belonging to GI (AB510015) was also detected in a secondary-treated wastewater sample (Fig. 1). Both GI and GIV genotypes were found in water samples, but only GIV was detected in oysters and human fecal specimens. The prevalence of SaV GIV infections in Japan during the investigation period might explain the frequent detection of this genotype in human feces in this study. The reason for only GIV being detected in oysters is not clear, but differences between SaV genotypes in the efficiency of accumulation in oysters should be investigated in a future study.

To our knowledge, this is the first study to report detection of human SaV in commercial oysters that meet the legal requirements for raw consumption in Japan. Furthermore, our results demonstrate that SaV can accumulate in oysters which are cultivated in an estuary receiving treated wastewater. The presence of very similar or identical nucleotide sequences of SaV GIV in fecal specimens from gastroenteritis patients, wastewater, cultivated oysters, and oysters for raw consumption during the same epidemiological season implies that SaV excreted in feces are released into environmental water, and can contaminate commercial oysters.

The contribution of effluent from wastewater treatment plants and septic tanks to viral contamination of water environments has been reported for several enteric viruses, including norovirus (12, 14, 15). Our investigation shows that wastewater effluent can also lead to SaV contamination. The importance of virus removal during wastewater treatment processes, especially in regions adjacent to oyster cultivation areas, demands investigation in order to prevent contamination of cultivated oysters with enteric viruses. Furthermore, at present enforcement of food sanitation regulations in Japan focus mainly on bacterial contamination. Although further studies are needed in order to determine the health risks associated with eating virus-contaminated oysters, regulations and standards may need to be revised in order to address the problem of viral contamination of oysters. Furthermore, co-contamination with norovirus, a major causative agent of acute gastroenteritis, also should be included in a future study to discuss the regulations regarding oyster cultivation.

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Detection of Sapoviruses and Noroviruses in an Outbreak of Gastroenteritis Linked Genetically to Shellfish

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Norovirus (NoV) and sapovirus (SaV) are important pathogens of human gastroenteritis. Compared to NoV, the transmission route of SaV is unclear. An outbreak of gastroenteritis occurred at a restaurant in June 2008, and SaV and NoV were detected in fecal specimens from 17 people who ate at the restaurant and one asymptomatic food handler and also in stripped shellfish and liquids remaining in the shellfish packages by reverse transcription-polymerase chain reaction (RT-PCR) and/or real-time RT-PCR. Nucleotide sequencing analysis of the RT-PCR products corresponding to the partial capsid region revealed 99.3–100% identities for SaV and 98.6–99.3% identities for NoV among the digestive diverticulum of the frozen stripped shellfish (*Ruditapes philippinarum*), "Asari," the package liquid, and feces from symptomatic or asymptomatic guests. These results suggested a link between the consumption of contaminated shellfish and clinical features in the patients. While the transmission of NoV by shellfish has been reported, this report shows that SaV can also be transmitted by shellfish. ***J. Med. Virol. 82:1247–1254, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: norovirus; sapovirus; shellfish; gastroenteritis; phylogenetic analysis

INTRODUCTION

Norovirus (NoV) and sapovirus (SaV) are important viral causes of human gastroenteritis. NoV and SaV are non-enveloped viruses, with positive-sense, single-strand RNA molecules. NoV and SaV are composed of many genetically distinct strains [Farkas et al., 2004; Zheng et al., 2006]. NoV can be divided into five major genogroups, GI, GII, GIII, GIV, and GV, of which GI, GII, and GIV have been detected in humans. SaV can be divided into at least five major genogroups, GI, GII, GIII, GIV, and GV, of which GI, GII, GIV, and GV have been detected in humans. NoV and SaV cannot be propagated in cell culture. Therefore, reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR have been used widely for detection of these viruses in clinical and environmental specimens [Harada et al., 2009; Iwai et al., 2009]. Nucleotide sequencing is also used widely for molecular characterization of NoVs and SaVs from clinical specimens.

A number of outbreaks of gastroenteritis associated with NoV or SaV have been reported [Schvoerer et al., 1999; Cheesbrough et al., 2000; Inouye et al., 2000; Kageyama et al., 2004; Bon et al., 2005; Johansson et al., 2005; Blanton et al., 2006; Gallay et al., 2006; Wu et al., 2006; Svraka et al., 2007; Shinkawa et al., 2008; Wu et al., 2008; Pang et al., 2009]. NoV and SaV have been detected by RT-PCR in fecal samples from both symptomatic and asymptomatic persons [Ozawa et al., 2007; Motomura et al., 2008; Iwakiri et al., 2009; Yoshida et al., 2009], vomit samples of infected persons [Chadwick and McCann, 1994; Marks et al., 2000], environmental water [Ueki et al., 2005; Hansman et al., 2007c; Aw et al., 2009], and shellfish [Nishida et al., 2003; Costantini et al., 2006; Hansman et al., 2007a; Hansman et al., 2008]. Food-borne outbreaks of acute gastroenteritis associated with NoV and SaV have been reported [Noel et al., 1997; Girish et al., 2002; Johansson

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et al., 2002; Le Guyader et al., 2004; Friedman et al., 2005; Gallimore et al., 2005; Usuku et al., 2008]. However, there are some reports showing a direct link between patients and suspected food as vehicles for NoV gastroenteritis [Daniels et al., 2000; Kobayashi et al., 2004; Nenonen et al., 2009] and there are no such reports for SaV gastroenteritis.

The aims of this study were to test for NoV and SaV in stool specimens collected from subjects in a shellfish-associated outbreak of gastroenteritis and from the same batch of suspected shellfish as food vehicles and to conduct genetic characterization of any NoV and SaV strains. Nucleotide sequencing of the RT-PCR products corresponding to the partial capsid gene revealed the presence of very similar or identical viral nucleotide sequences in the fecal specimens from the patients and the suspected shellfish.

MATERIALS AND METHODS

Fecal Specimens

Fecal specimens were collected from 23 restaurant guests with symptoms of gastroenteritis and an additional person in 3 groups (A, B, and D) and from 10 asymptomatic food handlers. The specimens were stored at −80°C until use.

Shellfish Samples

Frozen stripped shellfish prepared from *Ruditapes philippinarum* and the liquids from shellfish (package liquid) remaining in three 1-kg packages of the shellfish were tested after storage at −80°C. The shellfish and package liquids were collected from the same batch as that used for the suspected menu item (undercooked clam au gratin).

RNA Extraction and cDNA Synthesis

Nucleic acids were extracted from 100 µl of a 10% (w/v) stool suspension with an SV total RNA Isolation System (Promega, Tokyo, Japan) according to the manufacturer's protocol. The digestive diverticulum of the stripped shellfish (designated as Asari-DT) was prepared using a previously described method [Ueki et al., 2005] with some modifications. Briefly, the digestive diverticula of 20 stripped shellfish were removed by dissection and 1 g of the digestive diverticula was homogenized with an Eppendorf micropestle (Eppendorf, Tokyo, Japan) in 1 ml of phosphate-buffered saline without magnesium and calcium (PBS [−]); then, 25 mg of α-amylase (Wako Pure Chemical Industries, Osaka, Japan) was added, and the mixture was incubated at 37°C for 1 h. After centrifugation at 8,700*g* for 7 min, the supernatant was used for RNA extraction. Package liquid was collected from each of three thawed shellfish packages, and 40 ml was centrifuged at 6,000*g* for 20 min, and then the supernatant was layered onto 2 ml of 30% sucrose solution and ultracentrifuged in a P40ST rotor at 40,000 rpm for 2 h at 4°C (Hitachi Koki, Tokyo, Japan, Himac CP 80α). The pellet was resuspended in 560 µl of distilled water and used for RNA extraction. Nucleic acids were extracted with a QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and then eluted in 60 µl elution buffer according to the manufacturer's protocol. The RNA solution was treated with 1 U of RNase-free DNase I (Takara, Shiga, Japan) for 10 min at 37°C, after which the enzyme was inactivated by treatment at 75°C for 5 min. RT was performed with 10 µl of RNA solution with or without DNase I treatment and 30 µl of RT mixture containing 2 mM dNTPs mixture, 20 mM dithiothreitol, 0.3 µg of random nonamer primer (Takara), 1 µg of oligo pd(T)$_{12-18}$ (Invitrogen, Tokyo, Japan), 40 U of RNase inhibitor (Takara), 400 U of M-MLV reverse transcriptase (Invitrogen) and 8 µl of 5 × first-strand buffer (Invitrogen).

Real-Time PCR and Nested PCR

For SaV, MGB TaqMan®-based real-time PCR, which detects all human genogroups, and nested PCR for SaV were carried out as previously described [Oka et al., 2006; Okada et al., 2006]. Primers F13, F14, R13, and R14 were used for the first PCR, and then primers F22 and R2 were used for the nested PCR. In real-time PCR, samples were determined to be positive for SaV when the copy number in 5 µl of cDNA template was greater than 25 using standard plasmid DNA [Oka et al., 2006]. For NoV, genogroup-specific TaqMan-based real-time PCR and genogroup-specific semi-nested PCR were carried out as previously described [Kageyama et al., 2003; Hansman et al., 2008]. For genogroup I (GI), COG1F and G1SKR primers [Kojima et al., 2002; Kageyama et al., 2003] were used for the first PCR, and then G1SKF and G1SKR primers [Kojima et al., 2002] were used for the nested PCR. For NoV genogroup II (GII), COG2F and G2SKR primers [Kojima et al., 2002; Kageyama et al., 2003] were used for the first PCR, and then G2SKF and G2SKR primers [Kojima et al., 2002] were used for the nested PCR. In real-time PCR, samples were determined to be positive for NoV when the copy number in 5 µl of cDNA template was greater than 10 using a standard plasmid DNA [Nishida et al., 2003].

The cDNA prepared from DNaseI-treated RNA was used for the detection of NoV in human feces, the digestive diverticulum of stripped shellfish and the package liquid and for detection of SaV in feces. To detect the SaV genome from the digestive diverticulum of stripped shellfish or the package liquid, cDNA prepared from RNA not treated with DNase I was used as a template, and the number of cycles for nested PCR was increased from 35 to 50.

Nucleotide Sequencing and Phylogenetic Analysis

The PCR products amplified with the F22 and R2 primer set for SaV, the COG1F and G1SKR primer set for NoV GI from stools, the G1SKF and G1SKR primer set for NoV GI from clams, the COG2F and G2SKR

primer set for NoV GII from stools, and the G2SKF and G2SKR primer set for NoV GII from clams were excised from the gel and purified by a QIAquick gel extraction kit (Qiagen) or a QIAquick PCR purification kit (Qiagen) and then cloned into a pCR2.1 vector (Invitrogen). The insert was amplified with M13-RV primer (5′-CAGGAAACAGCTATGAC-3′) and M13-20 primer (5′-GTAAAACGACGGCCAG-3′), and the PCR products were separated by 2% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen), and at least four clones from each specimen were sequenced directly with M13-20 and -RV primers by a 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan) with a Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems). Nucleotide sequences were assembled with SEQUENCHER version 4.7 (Gene Codes Corporation, Ann Arbor, MI) and aligned with Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html). A representative sequence(s) from each specimen was used for a homology search (BLAST) and phylogenetic analysis. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method. The distance of the nucleotide substitutions per site was calculated by Kimura's two-parameter method and illustrated using NJPlot software (http://pbil.univ-lyon1.fr/software/njplot.html).

Nucleotide Sequence Accession Numbers

The SaV and NoV nucleotide sequences determined in this study have been deposited in DDBJ under the accession numbers AB522390-AB522424.

RESULTS

Description of Outbreak

An outbreak of gastroenteritis occurred among restaurant guests (groups A, B, and C) on June 6 and guests of a party (group D) who ate catered food on June 7, 2008. Thirty-eight (76%) of the 50 guests had clinical symptoms associated with gastroenteritis. The attack rates were 75% (21 of 28) in group A, 87.5% (7 of 8) in group B, 67% (2 of 3) in group C, and 72.7% (8 of 11) in group D. None of the 10 food handlers who worked at the restaurant had gastroenteritis symptoms.

Thirty-four stool specimens were collected from the 60 people (50 guests and 10 food handlers), including 23 specimens from symptomatic guests (13 in group A, 5 in group B, and 5 in group D), one specimen from an asymptomatic guest in group A, and 10 specimens from asymptomatic food handlers (Table I). Stool specimens were not collected from group C. The symptoms of 23 symptomatic guests available for pathogen screening were diarrhea (78.3%), vomiting (60.9%), abdominal pain (60.9%), and fever (higher than 37.5°C) (26.1%) (Table I). The incubation periods were from 5 to 60.5 h (median, 36.0 h) (Table I).

Through interviews with the guests by food inspectors, salmon and clam au gratin were identified as the common foods in groups A, B, and C; the latter was suspected to be a source of this outbreak because the members of groups A, B, and C claimed that the onion in the clam au gratin was undercooked. While the members of group D did not eat the clam au gratin, the catered food for group D was prepared on the same cooking counter on June 6 and stored in a refrigerator. In the restaurant, imported frozen stripped shellfish was used to prepare the clam au gratin (5 stripped shellfish per one clam au gratin). Based on this information, it was suspected that the gastroenteritis outbreak in groups A, B, and C occurred from the consumption of undercooked shellfish and that the outbreak in group D occurred from the consumption of some food(s) which were contaminated during preparation in the same kitchen. The fecal specimens were screened initially for enteropathogenic bacterial pathogens, *Salmonella* spp., *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Campylobacter* spp., *Vibrio cholerae*, and *Shigella* spp.; however, all specimens were negative (data not shown).

Virus Detection in Fecal Specimens From Patients and Food Handlers

Of 24 fecal specimens collected from the guests, 12 were positive for SaV (including a specimen from an asymptomatic guest, D1729) by nested RT-PCR, and 11 were positive for NoV by semi-nested RT-PCR, of which 9 were GI and 4 were GII (2 positive for both GI and GII). Five patients, D1739 in group A, D1714 in group B, and D1734, D1741, and D1743 in group D, were positive for both SaV and NoV (Table II). Thirteen guests (D1711, D1712, D1729, D1730, D1731, D1732, D1736, D1737, and D1738 from group A, D1715, D1716, and D1718 from group B, and D1742 from group D) were positive for just one virus, NoV or SaV (Table II). The numbers of viral cDNA copies per gram of fecal specimen determined by real-time RT-PCR ranged from 3.46×10^5 to 2.09×10^{10} for SaV, 4.4×10^6 to 2.0×10^7 for NoV GI and 9.6×10^6 to 3.9×10^9 for NoV GII (Table II). One of the 10 asymptomatic food handlers (D1725) was positive for NoV GII by both semi-nested PCR and real-time PCR (1.4×10^6 copies per gram of fecal specimen) (Table II).

Virus Detection in Stripped Shellfish and the Package Liquid

SaV and NoV were detected by PCR from the shellfish and the package liquids collected from three individual stripped shellfish packages (Package-1, -2, and -3) (Table II). The shellfish from Package 1 were positive for both SaV and NoV GII by RT-nested PCR and/or real-time RT-PCR. The liquid from Package 1 was also positive for both SaV and NoV GI. The liquid from Package 2 was positive for SaV by nested RT-PCR and the digestive diverticulum from Package 3 was positive for NoV GI by semi-nested RT-PCR (Table II). SaV in stripped shellfish or the package liquid was positive only

TABLE I. Description of Guests and Food Handlers Associated With the Outbreak

Group	Sample ID	Onset of illness	Incubation period (hr)	Specimen collected date	Sex	Age	Diarrhea	Vomiting nausea	Abdominal pain	Fever[a]
A	D1710	June 7, 2008	11	June 10, 2008	M	54	−	+	−	−
	D1711	June 8, 2008	37	June 10, 2008	F	47	+	+	+	−
	D1712	June 7, 2008	23	June 10, 2008	M	27	+	+	+	+
	D1713	June 8, 2008	49	June 10, 2008	M	57	+	−	+	−
	D1729	—	—	June 12, 2008	F	31	−	−	−	−
	D1730	June 7, 2008	5	June 11, 2008	F	42	−	−	+	−
	D1731	June 8, 2008	47	June 12, 2008	F	27	+	+	+	+
	D1732	June 8, 2008	38	June 12, 2008	F	21	+	−	+	−
	D1735	June 7, 2008	13	June 13, 2008	F	37	+	+	−	−
	D1736	June 8, 2008	34.5	June 13, 2008	F	40	+	+	−	−
	D1737	June 8, 2008	37	June 13, 2008	M	25	+	+	+	+
	D1738	June 7, 2008	14	June 13, 2008	F	29	+	−	+	−
	D1739	June 8, 2008	43.5	June 13, 2008	F	27	+	+	−	−
	D1740	June 8, 2008	36	June 13, 2008	F	22	+	−	−	−
B	D1714	June 9, 2008	60.5	June 10, 2008	M	58	+	−	−	+
	D1715	June 8, 2008	49.5	June 10, 2008	M	56	+	−	+	−
	D1716	June 7, 2008	13.5	June 10, 2008	M	—	+	+	+	−
	D1717	June 7, 2008	13.5	June 10, 2008	M	59	+	−	+	+
	D1718	June 8, 2008	43.5	June 10, 2008	M	59	−	−	+	−
D	D1733	June 8, 2008	34	June 12, 2008	M	—	+	+	−	−
	D1734	June 9, 2008	40.5	June 12, 2008	F	—	−	+	−	−
	D1741	June 8, 2008	33	June 13, 2008	F	—	+	+	+	−
	D1742	June 9, 2008	36	June 13, 2008	M	—	+	+	+	−
	D1743	June 9, 2008	43	June 13, 2008	F	—	−	+	−	+
Food handler	D1719	—	—	June 10, 2008	M	51	−	−	−	−
	D1720	—	—	June 10, 2008	M	49	−	−	−	−
	D1721	—	—	June 10, 2008	M	30	−	−	−	−
	D1722	—	—	June 10, 2008	M	28	−	−	−	−
	D1723	—	—	June 10, 2008	M	38	−	−	−	−
	D1724	—	—	June 10, 2008	M	34	−	−	−	−
	D1725	—	—	June 10, 2008	M	57	−	−	−	−
	D1726	—	—	June 10, 2008	M	26	−	−	−	−
	D1727	—	—	June 11, 2008	M	59	−	−	−	−
	D1728	—	—	June 11, 2008	M	52	−	−	−	−

[a]Higher than 37.5°.

when cDNA prepared from nucleic acid without DNase I treatment was used as a template for RT-PCR and/or RT-real-time PCR (data not shown).

Phylogenetic and Nucleotide Sequence Analyses

Nucleotide sequences of 8 GI SaV, 9 GII SaV, 12 GI NoV, and 6 GII NoV strains were divided into three, two, two, and five different branches, respectively, when their phylogenetic trees were constructed by NJplot analysis based on the partial capsid nucleotide sequences (Figs. 1 and 2). Among these branches, there were three clusters for SaVs (2 GI clusters and 1 GII cluster: cluster A including D1729-A, D1743-D-a, and Package 1-Liquid, cluster B including D1736-A, D1734-D, and Asari1-DT-a, and cluster C [the GII cluster] including D1737-A, D1739-A, D1743-D-b, Asari1-DT-b, and Package 2-Liquid), one cluster for NoV GI designated as cluster A (D1712-A, D1730A, D1731-A, D1739-A, D1715-B, D1716-B, D1734-D, D1741-D-b, D1742-D, Package 1-Liquid, and Asari3-DT), and one cluster for NoV GII designated as cluster B (D1714-B and Asari1-DT). The nucleotide sequence identities of the PCR products for SaV (405 bp) between patients and digestive diverticulum and/or package liquid were 100% in 2 GI clusters (designated as clusters A and B) and 99.3–99.8% (1–3 nucleotide difference) in one GII cluster (designated as cluster C) for SaV. Similarly, they were 98.6–99.7% (1–4 nucleotide difference) and 99.3% (2 nucleotide difference) in the NoV GI cluster (designated as cluster A) and the NoV GII cluster (designated as cluster B), respectively, when 295 or 282 bp were analyzed, respectively. These results showed very similar or identical nucleotide sequences among the fecal specimens from the symptomatic or asymptomatic guests and digestive diverticulum and/or the liquid collected from the frozen stripped shellfish packages.

DISCUSSION

It has been suspected that SaV can be transmitted by shellfish and cause subsequently outbreaks of gastroenteritis because SaVs were detected from shellfish which were purchased from markets [Hansman et al., 2007a] and also detected in stool specimens that were collected from gastroenteritis patients associated with the consumption of shellfish [Nakagawa-Okamoto

TABLE II. Detection of Norovirus and Sapovirus in Fecal Specimens From Guests, Food Handlers, and From Shellfish Specimens

		Sapovirus				Norovirus GI				Norovirus GII			
Group	Sample ID	Viral load[a]	Nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.
A	D1710	—	–			—	–			—	–		
	D1711	1.90×10^{8}	+	D1711-A/Jun2008/JPN_2	AB522391	—	–			—	–		
	D1712	—	–			1.2×10^{7}	+	D1712-A-a/Jun2008/JPN_1	AB522407	9.6×10^{6}	+	D1712-A-b/Jun2008/JPN_13	AB522419
	D1713	—	–			—	–			—	–		
	D1729	2.09×10^{10}	+	D1729-A/Jun2008/JPN_4	AB522393	—	–			—	–		
	D1730	—	–			2.5×10^{6}	+	D1730-A/Jun2008/JPN_5	AB522411	—	–		
	D1731	—	–			9.6×10^{6}	+	D1731-A/Jun2008/JPN_6	AB522412	—	–		
	D1732	1.23×10^{7}	+	D1732-A/Jun2008/JPN_5	AB522394	—	–			—	–		
	D1735	—	–			—	–			—	–		
	D1736	6.70×10^{9}	+	D1736-A/Jun2008/JPN_7	AB522396	—	–			—	–		
	D1737	1.21×10^{10}	+	D1737-A/Jun2008/JPN_8	AB522397	—	–			—	–		
	D1738	7.42×10^{8}	+	D1738-A/Jun2008/JPN_9	AB522398	—	–			—	–		
	D1739	2.44×10^{5}	+	D1739-A/Jun2008/JPN_10	AB522399	1.2×10^{7}	+	D1739-A/Jun2008/JPN_8	AB522414	—	–		
	D1740	—	–			—	–			—	–		
B	D1714	—	+	D1714-B/Jun2008/JPN_1	AB522390	—	–			1.3×10^{8}	+	D1714-B/Jun2008/JPN_14	AB522420
	D1715	—	–			2.0×10^{7}	+	D1715-B-a/Jun2008/JPN_2 D1715-B-b/Jun2008/JPN_3	AB522408 AB522409	—	–		
	D1716	—	–			2.0×10^{7}	+	D1716-B/Jun2008/JPN_4	AB522410	—	–		
	D1717	—	–			—	–			—	–		
	D1718	3.50×10^{8}	+	D1718-B/Jun2008/JPN_3	AB522392	—	–			—	–		
D	D1733	—	–			—	–			—	–		
	D1734	3.24×10^{8}	+	D1734-D/Jun2008/JPN_6	AB522395	2.0×10^{7}	+	D1734-D/Jun2008/JPN_7	AB522413	—	–		
	D1741	3.87×10^{7}	+	D1741-D/Jun2008/JPN_11	AB522400	—	+	D1741-D-a/Jun2008/JPN_9	AB522415	3.9×10^{9}		D1741-D-b/Jun2008/JPN_16	AB522422
	D1742	—	–			4.4×10^{6}	+	D1742-D/Jun2008/JPN_10	AB522416	—	–		
	D1743	3.46×10^{5}	+	D1743-D-a/Jun2008/JPN_12 D1743-D-b/Jun2008/JPN_13	AB522401 AB522402	—	–			1.5×10^{9}	+	D1743-D/Jun2008/JPN_17	AB522423
Food handler	D1719	—	–			—	–			—	–		
	D1720	—	–			—	–			—	–		
	D1721	—	–			—	–			—	–		
	D1722	—	–			—	–			—	–		
	D1723	—	–			—	–			—	–		
	D1724	—	–			—	–			—	–		

(*Continued*)

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TABLE II. (*Continued*)

Group	Sample ID	Sapovirus				Norovirus GI				Norovirus GII			
		Viral load[a]	Nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.
	D1725	—	—			—	—			1.4×10^6	+	D1725/Jun2008/JPN_15	AB522421
	D1726	—	—			—	—			—	—		
	D1727	—	—			—	—			—	—		
	D1728	—	—			—	—			—	—		
Ruditapes philippinarum (Asari)													
Package 1	DT 1[b]	+	+	Asari1-DT-a/Jun2008/JPN_14	AB522403	—	—			+	+	Asari1-DT/Jun2008/JPN_18	AB522424
				Asari1-DT-b/Jun2008/JPN_15	AB522404								
	Liquid 1	—	+	Asari1-Liquid/Jun2008/JPN_16	AB522405	—	+	Asari1-Liquid/Jun2008/JPN_12	AB522418	—	—		
Package 2	DT 2[b]	—	—			—	—			—	—		
	Liquid 2	—	+	Asari2-Liquid/Jun2008/JPN_17	AB522406	—	—			—	—		
Package 3	DT 3[b]	—	—			—	+	Asari3-DT/Jun2008/JPN_11	AB522417	—	—		
	Liquid 3	—	—			—	—			—	—		

[a]Number represents copies/g feces.
[b]DT indicates digestive diverticulum.

Fig. 1. Phylogenetic tree of SaV based on partial capsid nucleotide sequences. Seventeen SaVs detected in this study are shown in bold face on the tree. Clusters A and B in genogroup I and cluster C in genogroup II in this study are boxed. The numbers on each branch indicate the bootstrap values for the cluster, where values of 950 or higher were considered sufficiently significant for grouping. The scale indicates the nucleotide substitutions per site.

et al., 2009]. In this study, identical or very similar SaV nucleotide sequences (~99.3% nucleotide identity) were demonstrated on the basis of partial capsid nucleotide sequences in patients and shellfish from the suspected batch, similar to the case of NoV. The results strongly suggested food-to-human transmission for SaV in addition to NoV by shellfish cooked inadequately. However, further investigation is needed for determining the relation between SaVs found in shellfish and patients using variable region sequences such as the NoV P2 domain to show the identity of the genome.

SaV and NoV have been detected from feces of symptomatic patients with oyster-associated gastroenteritis; however, the details of clinical symptoms were not available [Nakagawa-Okamoto et al., 2009]. Therefore, it was interesting to determine whether multiple virus infections cause severe clinical symptoms. Clinical symptoms and the incubation periods of patients infected with multiple species of viruses were not different from those of patients infected with a single species of virus, although the number of patients compared was limited (Tables I and II). In addition, an

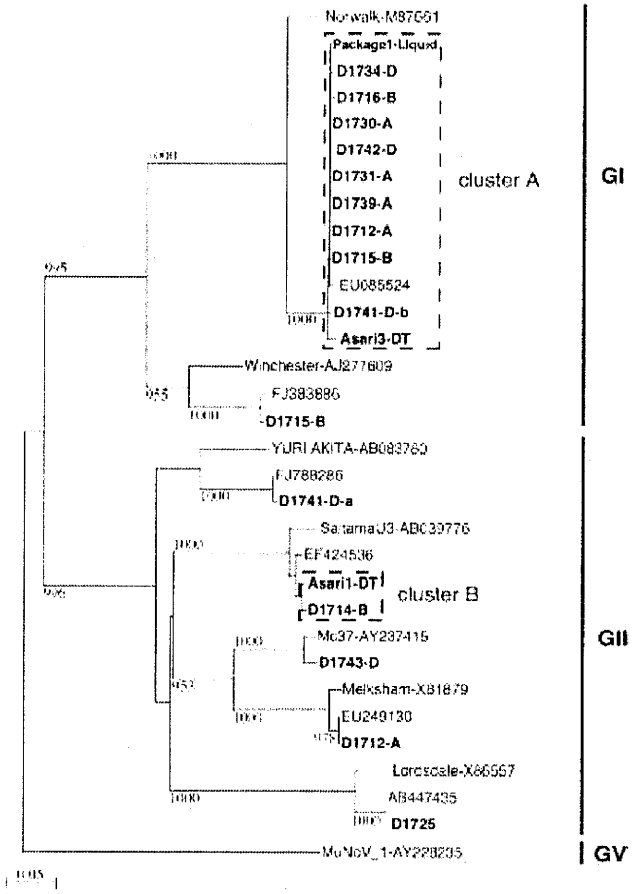

Fig. 2. Phylogenetic tree of NoV based on partial capsid nucleotide sequences. Eighteen NoVs detected in this study are shown in bold face on the tree. Cluster A in genogroup I and cluster B in genogroup II in this study are boxed. The numbers on each branch indicate the bootstrap values for cluster, where values of 950 or higher were considered sufficiently significant for grouping. The scale indicates the nucleotide substitutions per site.

asymptomatic guest (D1729) had the highest SaV RNA level among those providing fecal specimens (Tables I and II). These results suggest that there is no direct relation between combined infection of NoV and SaV and particular or severe gastroenteritis symptoms, although further investigation is necessary.

The presence of genetically diverse NoV and SaV strains in shellfish is well known. Indeed, NoV and SaV with various nucleotide sequences were detected in shellfish samples examined in this study (Figs. 1 and 2). The viral load of NoV and SaV in the stripped shellfish or the package liquid seemed to be low because it was mostly below the detection limit by real-time RT-PCR (Table I). Nevertheless, it is likely that SaV and NoV concentrated in shellfish or leaked into the package liquid are capable of transmission to humans and causing gastroenteritis.

Based on a BLAST search, Package 1-Liquid (cluster A), Asari1-DT-a (cluster B), and Package 2-Liquid (cluster C) for SaV (Fig. 1) were close to Yokote1 (AB253740) [Hansman et al., 2007b] detected in a 5-year-old female in 2006 in Japan, oyster/Miyagi/1/2009 (AB510019) detected from an oyster (*Crassostrea gigas*) in 2009 in Japan (AB510019), and Kumamoto6/2003 (AB429084) detected in a 1-year-old female in 2003 in Japan [Harada et al., 2009] with 99%, 98%, and 99% nucleotide identities, respectively, when approximately 350–400-nt partial capsid nucleotide sequences were compared (data not shown). Package 1-Liquid, belonging to cluster A for NoV GI (Fig. 2), was close to EU085524 detected from mussels (*Mytilus edulis*) in 2004 in Sweden [Nenonen et al., 2008], and Asari1-DT, belonging the cluster B for NoV GII (Fig. 2), was close to EF424536 detected from clams (*Corbicula japonica*) in 2006 in Japan [Hansman et al., 2008] when approximately 280–290-nt partial capsid nucleotide sequences were compared. These results suggest that genetically similar SaVs and NoVs are distributed in various areas of Japan and abroad.

While NoV GII sequence was detected from an asymptomatic food handler (D1725), the same sequence was not detected in any of the guests (Fig. 2). Therefore, the nucleotide sequence analysis clearly ruled out the possibility that this food handler was the source of infection in this case.

In conclusion, we detected SaV and NoV from patients with gastroenteritis and the suspected food (shellfish). To control SaV and NoV infection by intake of shellfish, their stability against heat and the criteria for viruses in shellfish and harvest areas should be clarified.

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