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Distribution of Marine Birnavirus (MABV) in Marine Organisms from Okinawa, Japan, and a Unique Sequence Variation of the VP2/NS Region

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Distribution of marine type of Aquabirnavirus (MABV) was examined in shellfish and fish from Okinawa and Ishigaki Islands, Japan, where water temperature is higher than 25°C through the year. Genome detection and virus isolation were performed for shellfish and fish samples, and the results revealed the prevalent distribution of MABV in diverse species in the area, although isolation was not frequently. Detection rate of MABV genome in bivalves was higher than gastropods, which was similar result to former report in mainland of Japan. Furthermore, the unique five-nucleotide deletion was found with a high rate of occurrence in the MABV genome from shellfish and fish. This study showed distribution status of MABV in organisms in subtropical waters by wide monitoring, and discovered new genome variation in VP2/NS region of this virus.

***Keywords*:** birnavirus, shellfish, marine, Okinawa

The marine type of Aquabirnavirus (marine birnavirus; MABV) belongs to the genus *Aquabirnavirus*, which is an independent genogroup to the infectious pancreatic necrosis virus (IPNV) infecting salmonid fishes (Dobos *et al*., 1979; Zhang and Suzuki, 2003). Aquabirnavirus has been detected in various kinds of aquatic organisms including vertebrates and invertebrates (Leong *et al*., 2000). For example, in fish MABV has been isolated from various species, including red sea bream *Pagrus major* (Kusuda *et al*., 1994), tiger puffer *Takifugu rubripes* (Kamakura *et al*., 1995), as well as yellowtail (*Seriola quinqueradiata*) (Sorimachi and Hara, 1985) which was originally reported for birnavirus disease in marine fish. Among MABV, isolates developing ascites in yellowtail have been called yellowtail ascites virus (YTAV, Egusa and Sorimachi, 1986). More recent reports have indicated that MABV infects olive flounder (Japanese flounder) (*Paralichthys olivaceus*) in Japan and Korea (Inaba *et al*., 2007; Jung *et al*., 2008). Despite findings that MABV shows pathogenicity in various fish species, the infection cycle of this virus in the environment remains unknown.

Shellfish are also known to be hosts of MABV (Suzuki *et al*., 1997b, 1998a) with weak pathogenicity (Suzuki *et al*., 1998b). Evidence reported from fish and shellfish indicate that the host range of MABV is extremely broad; however, the mechanism and pathway of infection, such as through various intermediate hosts, is unknown. MABV has also been detected from natural seawater (Kitamura and Suzuki, 2000; Kitamura *et al*., 2002; Suzuki *et al*., 2005), suggesting that MABV can exist in a cell-free form in the environment, although there has been no report on the production host of the cell-free MABV. From the report by Suzuki and Nojima (1999), it is clear that MABV is ubiquitously distributed in shellfish species of mainland Japan, suggesting that the shellfish are natural hosts that may release MABV into the environment. The released virion can retain infectivity in natural seawater for 170 min at 28°C (Kitamura *et al*., 2004), which may allow for adequate time for horizontal infection to other organisms. To clarify natural hosts of the virus is important in controlling the virus-borne diseases in cultured fish and shellfish. Characteristics of MABV described above strongly suggest the infection cycle in marine ecosystems occurs through water, shellfish and fish. Suzuki and Nojima (1999) indicated that marine shellfish are common hosts of MABV, suggesting that shellfish may act as potential reservoir of this virus prior to infecting cultured fish. Thus the monitoring of MABV in shellfish provides essential information on how to mitigate potential natural reservoirs of this virus.

IPNV has been cultured under temperatures <24°C (Dobos and Roberts, 1979); however, the shellfish birnavirus can be cultured at 30°C as well as 20°C (Lo *et al*., 1988). Infection by MABV and MABV-like birnaviruses has been reported from Taiwan, and Ehime and Kumamoto Prefectures of Japan, where seawater temperature can reach as high as 30°C (Chou *et al*., 1994; Suzuki *et al*., 1997b; Kitamura *et al*., 2000). Tolerance to such high temperatures is an advantage for MABV to expand its ecological niche across a broad range of environments. The natural distribution of MABV in subtropical waters is still unknown, however. In this study, we examined the distribution of MABV in subtropical waters of Okinawa, Japan, and discovered genetic variations of the MABV from fish and shellfish in this region.

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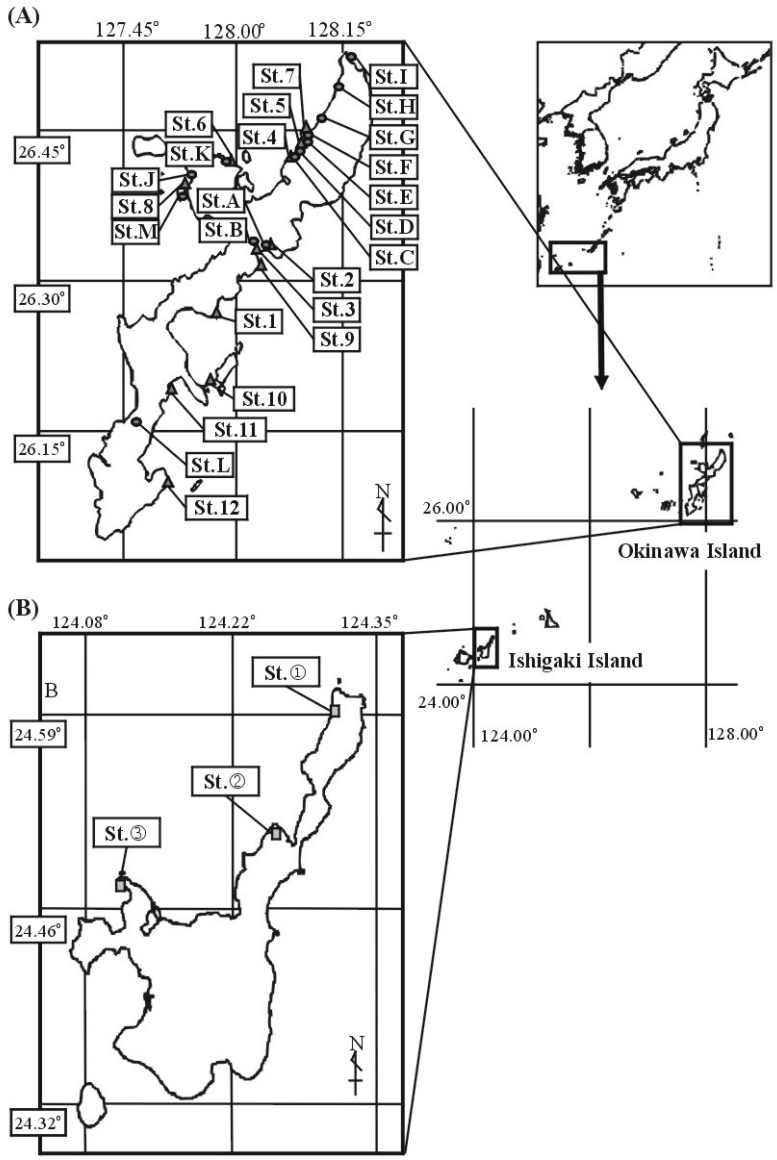

Fig. 1. Sampling sites in 2003 (St. 1~12), 2004 (St. A~M) and 2005 (St. ①~③). (A) Okinawa Island and (B) Ishigaki Island.

Materials and Methods

Sample

Sampling was performed on the main island of Okinawa, Japan, and Ishigaki Island which is an offset island west of Okinawa (hereafter named Okinawa and Ishigaki) (Fig. 1). Shellfish samples were collected in June and July in 2003 and July in 2004 in Okinawa, and July in 2005 in Ishigaki; Fish samples were collected in June in 2004 in Okinawa. Upon collection all samples were placed on ice and transported to the laboratory. The species and number of shellfish used for analysis are shown in Table 1. All samples were applied to a birnavirus-specific PCR assay (Suzuki *et al*., 1997a), and samples that were virus genome-positive by PCR were also tested using a virus isolation technique.

Virus genome detection

A two-step PCR technique was performed to detect the birnavirus genome from shellfish and fish samples (Suzuki *et al*., 1997a). For shellfish samples, all organs except for muscular tissue were taken and used for virus detection. In the case of fish samples, all internal organs except for the intestine were used. Organs from five individuals were pooled as one specimen, which were homogenized with a plastic pestle in a 9-fold volume of 10 mM Tris-HCl (pH 8.0) – 1 mM EDTA buffer (TE), followed by centrifugation at 6,000×g for 5 min. A 45 μl portion of the supernatant was used for nucleic acid extraction. Five microliter of preteinase K (1 mg/ml, TaKaRa) was added to supernatant, which was then incubated at 55°C for 2 h. Nucleic acid was extracted with the phenol-chloroform-*iso* amyl alcohol (25:24:1, v/v/v) three times followed by ethanol precipitation. The extracted nu-

Table 1. Summary of samples examined

Site	Date	Water temperature (°C)	Species	No. of specimen
1	2003 Jun. 30	NM	*Cypraea annulus*[a]	6
2	2003 Jun. 30	NM	*Cypraea annulus*[a]	6
			Nerita albicilla[a]	5
			Ruditapes philippinarum[b]	6
3	2003 Jun. 30	NM	*Clithon sowerbianus*[a]	5
4	2003 Jun. 30	NM	*Ruditapes philippinarum*[b]	5
5	2003 Jun. 30	NM	*Theoduxus corona*[a]	5
			Clithon sowerbianus[a]	5
			Theodoxus retropictus[a]	5
			Nerita plumbea[a]	3
6	2003 Jul. 1	NM	*Cerithideopsillar cingulata*[a]	5
			Katelysia japonica[b]	5
			Gafrarium tumidum[b]	3
7	2003 Jul. 1	NM	*Theodoxus retropictus*[a]	5
8	2003 Jul. 1	29.8	*Conus ebraenus*[a]	5
			Cypraea caputserpentis[a]	2
			Cypraea annulus[a]	1
			Conus miles[a]	1
			Thais savignyi[a]	2
			Turbo setosus[a]	1
			Trochus maculatus[a]	1
			Tridacna crocea[b]	1
			Modiolus nipponicus[b]	2
			Fimbria fimbriata[b]	1
9	2003 Jul. 1	32.2	*Nerita albicilla*[a]	5
			Thais savignyi[a]	1
			Barbatia foliata[b]	5
			Modiolus nipponicus[b]	1
10	2003 Jul. 2	29.0	*Turbo coronatus coreensis*[a]	1
			Barbatia foliata[b]	5
			Gafrarium tumidum[b]	5
11	2003 Jul. 2	34.0	*Pinna bicolor*[b]	1
			Pharaonella perna[b]	1
			Trachycardium flavum[b]	1
			Modiolus nipponicus[b]	1
			Modiolus metacalfei[b]	3
12	2003 Jul. 3	31.8	*Nerita costata*[a]	5
			Isognomon ephippium[b]	5
			Ruditapes philippinarum[b]	1
A	2004 Jul. 3	30.2	*Nerita albicilla*[a]	7
			Monodonta labio[a]	9
			Monodonta australis[a]	3
B	2004 Jul. 3	31.0	*Clithon sowerbianus*[a]	6
			Batillaria multifarmis[a]	6
			Telebralia sulcata[a]	5
C	2004 Jul. 3	30.5	*Monodonta labio*[a]	3
			Nerita albicilla[a]	5
D	2004 Jul. 3	32.0	*Nerita plicata*[a]	2
			Nerita polita polita[a]	2
			Ruditapes philippinarum[b]	4
E	2004 Jul. 3	28.2	*Clithon sowerbianus*[a]	5
			Theodoxus corona[a]	5
			Theodoxus retropictus[a]	5
F	2004 Jul. 3	29.0	*Theodoxus retropictus*[a]	5
			Septaria porcellana[a]	5
G	2004 Jul. 3	34.0	*Trochus maculatus*[a]	5
			Turbo stenogyrus[a]	6
H	2004 Jul. 3	29.0	*Clithon sowerbianus*[a]	5
			Theodoxus corona[a]	5
			Theodoxus retropictus[a]	5
			Septaria porcellana[a]	4
I	2004 Jul. 3	29.0	*Theodoxus retropictus*[a]	5
			Clithon sowerbianus[a]	4
J	2004 Jul. 4	25.0	*Theodoxus retropictus*[a]	5
			Clithon sowerbianus[a]	4
K	2004 Jul. 4	29.0	*Gafrarium tumidum*[b]	2
			Katelysia japonica[b]	5
			Isognomon ephippium[b]	5
			Crassostrea nippona[b]	3
L	2004 Jul. 4	NM	*Achatina fulica*[a]	3
M	2004 Jul. 4	NM	*Apogon lineatus*[c]	8
			Halichoeres trimaculatus[c]	5
			Pomacentrus taeniometopon[c]	5
			Abudefduf sexfasciatus[c]	5
			Dascyllus trimaculatus[c]	5
①	2005 Jul. 22	31.0	*Atactodea striate*[b]	2
②	2005 Jul. 22	33.0	*Katelysia hiantina*[b]	6
			Gafrarium tumidum[b]	3
③	2005 Jul. 22	32.0	*Atactodea striate*[b]	7
			Mactra nipponica[b]	6
			Gafrarium tumidum[b]	4
			Katelysia striate[b]	3

NM, Not measured; [a] Gastropoda; [b] Bivalvia; [c] Fish

Table 2. Viral genome detection and virus isolation

Species	PCR positive[a] (%) in each year				Virus isolation[b]		
	2003	2004	2005	Total of three years	CHSE-214 (20°C)	EPC (20°C)	EPC (25°C)
Gastropoda							
Theodoxus retropictus	3/10 (30.0)	20/25 (80.0)	-	23/35 (65.7)	-	-	-
Clithon sowerbianus	0/10 (0)	15/24 (62.5)	-	15/34 (44.1)	-	-	-
Nerita albicalla	3/10 (30.0)	8/12 (66.7)	-	11/22 (50.0)	-	-	-
Theodoxus corona	2/5 (40.0)	7/10 (70.0)	-	9/15 (60.0)	-	-	-
Cypraea annulus	6/13 (46.2)	-	-	6/13 (46.2)	-	-	-
Monodonta labio	-	1/12 (8.3)	-	1/12 (8.3)	-	-	-
Septaria porcellana	-	3/9 (33.3)	-	3/9 (33.3)	-	-	-
Turbo stenogynus	-	3/6 (50.0)	-	3/6 (50.0)	-	-	-
Batillaria multiformis	-	2/6 (33.3)	-	2/6 (33.3)	-	-	-
Turbo coronatus coreensis	3/5 (60.0)	-	-	3/5 (60.0)	-	-	-
Nerita costata	3/5 (60.0)	-	-	3/5 (60.0)	-	-	-
Conus ebraenus	2/5 (40.0)	-	-	2/5 (40.0)	-	-	-
Terebralia sulcata	-	2/5 (40.0)	-	2/5 (40.0)	-	-	-
Trochus maculatus	-	1/5 (20.0)	-	1/5 (20.0)	-	-	-
Cerithideopsillar cingulata	0/5 (0)	-	-	0/5 (0)	-	-	-
Achatina flica	-	2/3 (66.7)	-	2/3 (66.7)	-	-	-
Monodonta asutralis	-	2/3 (66.7)	-	2/3 (66.7)	-	-	-
Thais savignyi	1/3 (33.3)	-	-	1/3 (33.3)	-	-	-
Nerita plumbea	0/3 (0)	-	-	0/3 (0)	-	-	-
Cypraea caputserpentis	2/2 (100)	-	-	2/2 (100)	1[c] ($1.0\times10^{3.57}$)[d]	-	-
Nerita plicata	-	1/2 (50.0)	-	1/2 (50.0)	-	-	-
Nerita polita polita	-	1/2 (50.0)	-	1/2 (50.0)	-	-	-
Turbo setosus	0/1 (0)	-	-	0/1 (0)	-	-	-
Conus miles	0/1 (0)	-	-	0/1 (0)	-	-	-
Trochus maculatus	0/1 (0)	-	-	0/1 (0)	-	-	-
Total	25/79 (31.7)	68/124(54.8)	-	93/203(45.8)			
Bivalvia							
Ruditapes philippinarum	4/11 (36.4)	2/4 (50.0)	-	6/15 (40.0)	-	-	-
Gafrarium tumidum	1/4 (25.0)	2/2 (100)	0/7 (0)	3/13 (23.1)	-	-	-
Barbatia foliata	8/10 (80.0)	-	-	8/10 (80.0)	-	-	1[c] ($1.0\times10^{4.15}$)[d]
Isognomon ephippium	3/5 (60.0)	3/5 (60.0)	-	6/10 (60.0)	-	-	-
Katelysia japonica	0/5 (0)	4/5 (80.0)	-	4/10 (40.0)	-	-	-
Katelysia hiantina	-	-	1/9 (11.1)	1/9 (11.1)	-	-	-
Atactodea striate	-	-	0/9 (0)	0/9 (0)	-	-	-
Modiolus nipponicus	5/6 (83.3)	-	-	5/6 (83.3)	-	-	-
Mactra nipponica	-	-	0/6 (0)	0/6 (0)	-	-	-
Modiolus metcalfei	3/3 (100)	-	-	3/3 (100)	-	-	-
Crassostrea nippona	-	1/3 (33.3)	-	1/3 (33.3)	-	-	-
Pharaonella perna	0/1 (0)	-	-	0/1 (0)	-	-	-
Fimbria fimbriata	0/1 (0)	-	-	0/1 (0)	-	-	-
Pinna bicolor	0/1 (0)	-	-	0/1 (0)	-	-	-
Tridacna crocea	0/1 (0)	-	-	0/1 (0)	-	-	-
Trachycardium flavum	-	-	-	0/1 (0)	-	-	-
Total	24/49 (49.0)	12/19 (63.2)	1/31 (3.2)	37/99 (37.4)			
Total of all shellfish species	49/128(38.3)	80/143(55.9)	1/31 (3.2)	130/302(43.0)			
Fish							
Apogon lineatus	-	2/8 (25.0)	-	2/8 (25.0)	-	-	-
Halichoeres trimaculatus	-	1/5 (20.0)	-	1/5 (20.0)	-	-	-
Pomacentrus taeniometopon	-	1/5 (20.0)	-	1/5 (20.0)	-	-	-
Abudefduf sexfasciatus	-	5/5 (100)	-	5/5 (100)	-	-	-
Dascyllus trimaculatus	-	4/5 (80.0)	-	4/5 (80.0)	-	-	-
Total	-	13/28 (46.4)	-	13/28 (46.4)			

[a] PCR positive number / specimen number; [b] result of 2nd inoculation; [c] number of virus isolation positive [d] infectivity titer in the supernatant of the 2nd inoculation ($TCID_{50}$/ml)

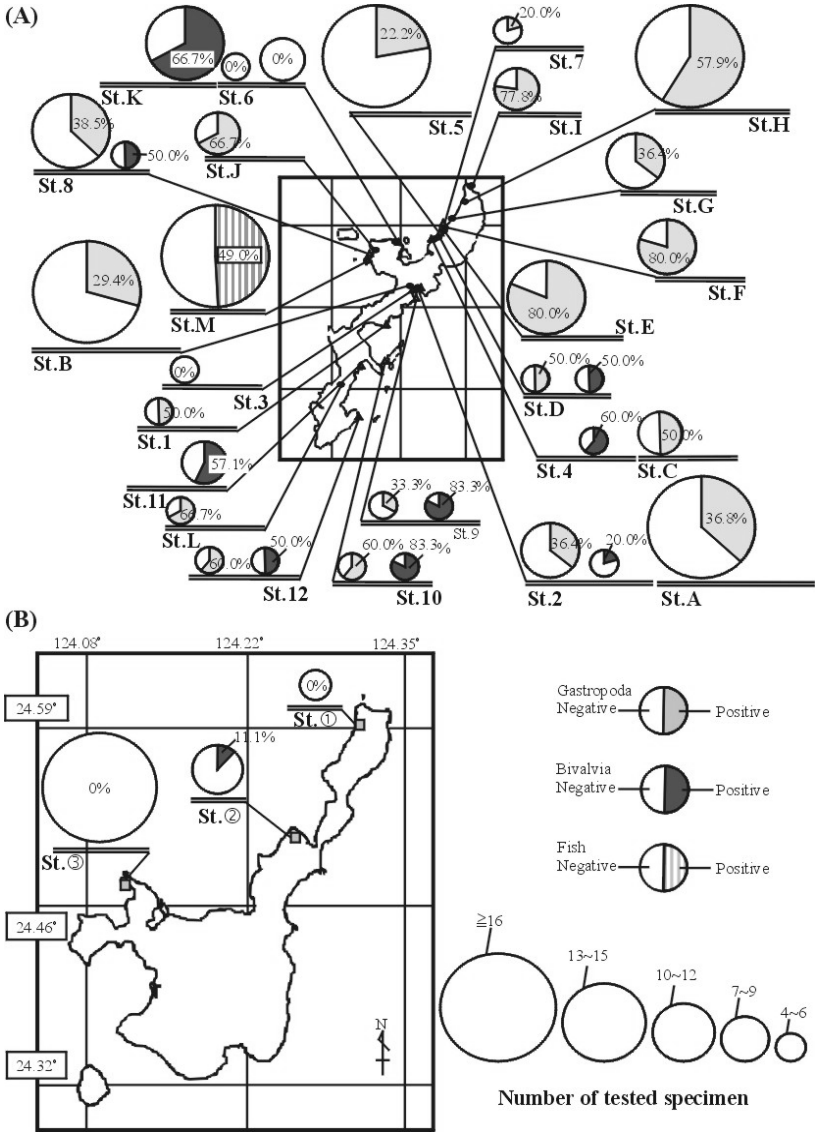

Fig. 2. Detection rates of the MABV genome from gastropod, bivalve and fish samples. In St. 6, small circle shows gastropods and medium circle shows bivalves. St. 3 shows gastropod data. St. ① and ③ are bivalve data.

cleic acid was heated at 100°C for 5 min, and a primer set of P1-P2 (P1; 5'-AGAGATCACTGACTTCACAAGTGAC-3', and P2; 5'-TGTGCACCACAGGAAAGATGACTC-3') was added to the nucleic acid solution. Reverse transcription (RT) reaction was performed by using MMLV reverse transcriptase (Invitrogen) at 37°C for 1 h, followed by heating at 100°C for 5 min. The PCR amplification in a DNA thermal cycler (Applied Biosystems) with *Taq* polymerase (TaKaRa) involved 30 cycles: 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. A 5 μl of the PCR product was used for nested-PCR, which was performed with 30 cycles with a primer set P3-P4 (P3; 5'-CAACACTCTTCCCCATG-3' and P4; 5'-AG AACCTCCCAGTGTCT-3'). The 10 μl of amplified nested-PCR products (168 bp) was visualized with ethidium bromide after electrophoresis on 2% agarose, at which the running buffer was 40 mM Tris-acetate (pH 8.0) and and 1 mM EDTA solution (TAE). The band clearly appeared at the same position of the PCR product of MABV Y-6 strain (Kusuda *et al*., 1993) was considered as positive. The indistinct band was confirmed by direct sequencing.

Virus isolation

Virus isolation was performed for all PCR-positive specimens. The chinook salmon embryo cell line (CHSE-214) and the epithelioma papulosum cyprine cell line (EPC) were used for isolation and quantitative analysis of the virus. Both cell lines were supplied by Professor M. Yoshimizu, Hokkaido University. Each cell line was grown at 20°C and the EPC was additionally grown at 25°C also. Eagle's minimums essential medium (MEM) containing 10% fetal bovine serum was used as the growth medium. The homogenates were inoculated onto each cell culture in a 24-well tissue-culture plate (Corning). The plates were inoculated for one week and cytopathic effect (CPE) was monitored daily. If CPE

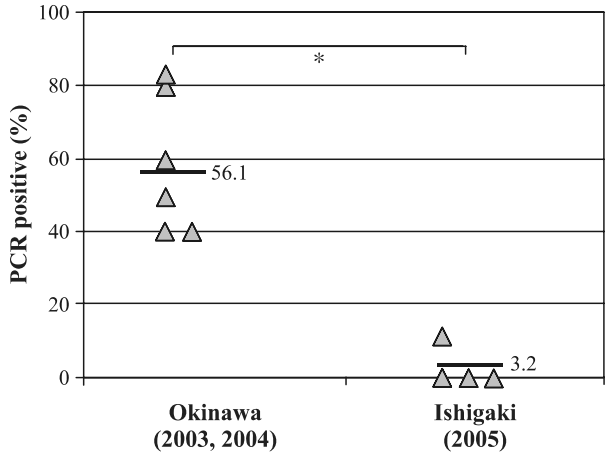

Fig. 3. Detection rate of the MABV genome in bivalves from Okinawa and Ishigaki islands. One triangle indicates one species. Six species in Okinawa and four species in Ishigaki are shown; each triangle denotes a species collection of having more than five specimens. One specimen is a composite of five individuals. Bar shows the average detection rate. Significant difference was calculated by paired *t*-test and significant difference (*) was obtained at $P<0.05$.

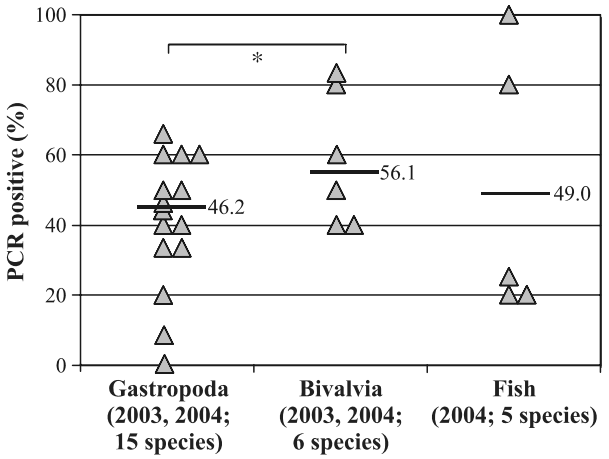

Fig. 4. Detection rate of the MABV genome in gastropods, bivalves, and fish from Okinawa. Symbols and data analysis are same as in Fig. 3. Bar shows average detection rate. Significant difference was calculated by paired *t*-test and significant difference (*) was obtained between gastropods and bivalves at $P<0.05$.

appeared, the supernatant was reinoculated to new cell culture in order to substantiate the presence of a virus. Virus-infectivity titration was performed by the 50% tissue culture infection dose ($TCID_{50}$) method, and the titer was calculated by the method of Reed and Muench (1938) using CHSE-214 in a 96-well tissue-culture plate (Corning).

Nucleotide sequencing

The nested-PCR product of MABV was shown as 168 bp long. Sequences in both strands of the PCR product from the shellfish and fish samples were determined in an automated DNA sequencer (310: Applied Biosystems) using an ABI PRISMTM Dye Terminator Cycle Sequencing Kit. The sequenced region contains the junction region of the VP2/NS in segment A (Suzuki *et al*., 1997a). This region is known to be variable, and differs from IPNV (Hosono *et al*., 1996). Analysis of the results was performed using Genetyx Win Version 5.1 software (Genetix).

Results

Distribution of the MABV genome in shellfish and fish samples

The MABV genome was detected from shellfish and fish of Okinawa and Ishigaki; results of genome detection from the samples are shown in Table 2. The total detection rate of MABV in shellfish samples from Okinawa in 2003 and 2004 were 38.3 and 55.9%, respectively, whereas that in shellfish from Ishigaki was 3.2%. Fish samples from Okinawa in 2004 showed 46.4% positive for MABV. The occurrence and distribution of the MABV genome from shellfish collected on Okinawa and Ishigaki are shown in Fig. 2. In both 2003 and 2004, the MABV genome was detected at most of the sampling sites in Okinawa (Fig. 2A) with two negative sites (St. 3 and 6) where sample sizes were small. Ishigaki (Fig. 2B) showed a low detection rate. Significant differences in detection rates of bivalves from Okinawa and Ishigaki were observed (Fig. 3). The water temperature measured at each sampling site was 25.0 to 34.0°C, with an average of 30.5°C (Table 1), indicating MABV was widely distributed in Okinawa where water temperatures were high throughout the year. The PCR positive rate depending on the host species shown in Fig. 4 indicates that the prevalence of MABV in bivalve species was greater than that in gastropods.

Virus isolation

Results of virus isolation for the 143 PCR-positive samples are listed in Table 2. Only one gastropod (*Cypraea coputserpentis*) and one bivalve species (*Barbatia foliate*) gave successful virus isolation in the 2nd passage. Conditions of the virus isolation were CHSE-214 cells at 20°C for *C. coputserpentis* and EPC cells at 25°C for *B. foliate*, respectively. The virus isolation rate in PCR positive samples was very low. The infectivity titers of each isolated virus were $1.0\times10^{3.57}$ $TCID_{50}$/g (*C. coputserpentis*) and $1.0\times10^{4.15}$ $TCID_{50}$/g (*B. foliate*).

Nucleotide sequences of PCR products and isolated virus

Sequence analysis was performed for the PCR products. Twenty-one PCR products were randomly chosen from shellfish samples of 2003. For 2004 and 2005 samples, all PCR products from shellfish (n=101) and fish (n=13) samples were analyzed. Comparison of sequences around 1617~1646th position of segment A of Y-6 strain and two shellfish cases is shown in Fig. 5. In Fig. 5, representative results of PCR products from gastropods are shown. Even at the same sampling site (St. H), different sequences were obtained. Five-nucleotide deletion (GGACT in Y-6 strain) was observed in 1630th to 1634th of the PCR product, and the occurrence rate of the deletion was 57.7% of gastropods and 87.0% of bivalves (Table 3). All fish samples showed

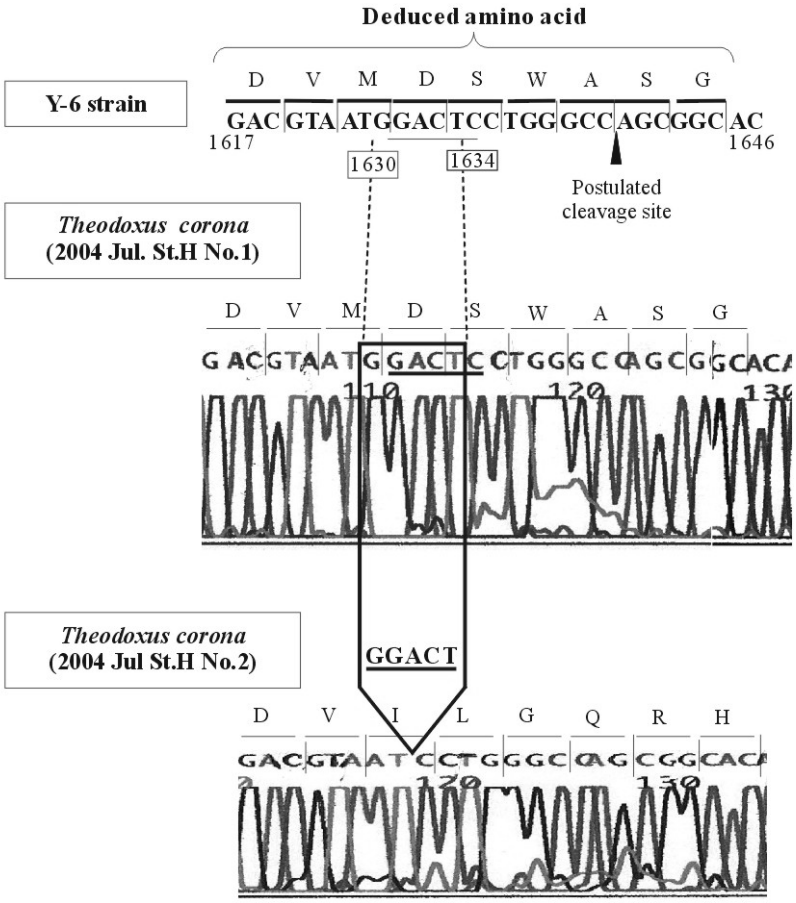

Fig. 5. Nucleotide and deduced amino acid sequences at the VP2/NS region of the PCR products and control virus strain Y-6. Y-6 strain and a gastropod (*Theodoxus corona* No. 1) had usual sequence, whereas *T. corona* No. 2 (same species but different individual) showed a five-nucleotide deletion. In the amino acid sequence, No. 2 indicates a change of amino acid when compared to the Y-6 sequence. Arrow shows the postulated cleavage site by protease.

the deletion, and this deletion resulted in an amino acid change around the NS protease cleaving site (Fig. 5). Both of the two isolated strains from gastropods and bivalves (Table 2) had the five-base deletion.

Discussion

The infection by MABV in marine organisms has been observed over a broad spatial distribution (Kusuda *et al*., 1994; Suzuki *et al*., 1997a; Suzuki *et al*., 1998a; Suzuki and Nojima, 1999; Jung *et al*., 2001) including cultured and wild shellfish in coastal areas of Japan and Korea. This suggests that the host range of MABV is very broad and natural reservoirs or carriers are also wildly distributed in the marine environment. MABV is typically cultured around 20°C~25°C under experimental condition; however, the shellfish birnavirus can be cultured at 30°C as well as 20°C (Lo *et al*., 1988). In this study, results from viral genome detection showed a prevalence of MABV from marine organisms in Okinawa and Ishigaki, where water temperature is higher than 25°C throughout the year.

Table 3. Occurrence of the five-nucleotide deletion

Category	Occurrence rate of the five-nucleotide deletion (%)
Gastropoda	45/78 (57.7)
Bivalvia	20/23 (87.0)
Fish	13/13 (100)

Over the two years of this study, MABV was detected from shellfish in most sampling sites of Okinawa, and there were no significant differences in detection rates between the individual sites. Moreover, it was shown that MABV exists in various shellfish species in Okinawa, as MABV was detected from 20 of the 25 gastropod species, and in 8 of the 18 bivalve species collected. This suggests that MABV exists widely across taxonomic groups of shellfish in Okinawa, and indicates the potential shellfish may play as carriers and/or reservoirs of MABV in subtropical waters of Okinawa. Shellfish may therefore have a substantial influence on the distribution of MABV. The detection rate of MABV in bivalves in Ishigaki was significantly lower than that in Okinawa (Fig. 3). In samples from mainland Japan, Suzuki, and Nojima (1999) reported that bivalves had MABV at a higher frequency than in gastropods; however, significant differ-

ences in detection rate were not found between the different environments. Although the cause of the significant difference between Okinawa and Ishigaki is unknown, Ishigaki may not be as contaminated with MABV compared to other areas. Current is moving from south (Ishigaki) to north (Okinawa) (Japan Meteorological Agency, http://www/jma.go.jp/index.html), which might affect on spreading of the virus.

The detection rate of MABV was compared between gastropods and bivalves, indicating a higher PCR detection rate in bivalve species (Fig. 4); this confirmed the findings by Suzuki and Nojima (1999). This study further found that the detection rate in fish was highly variable (20~100%) in the five species examined, and that there was no significant difference between fish and shellfish. Growth of MABV in various fish species is known to be variable in experimental infection (Isshiki *et al*., 2001). Although virus titers in each fish were not determined in this study, the variation of MABV detection rate in fish may be an artifact caused by the growth property of MABV in each species.

It was clear that MABV is distributed in many species of shellfish; however, the isolation rate was found to be very low. MABV has been considered to be an opportunistic pathogen in shellfish (Suzuki *et al*., 1998b; Kitamura *et al*., 2000). On the infection target in shellfish, Kitamura *et al*. (2000) found in Japanese pearl oysters that the virus persistently infected haemocyte tissue in summer, however, the virus replicated in haepatopancreas tissue in winter. Samples in Okinawa and Ishigaki in this study were collected in the summer season, thus suggesting that the virus has not replicated in shellfish. In general, to help hide from the host immune system the virus infects natural hosts without symptoms and maintains a low viral load. Given this case, our result from shellfish indicates that shellfish are likely natural hosts of MABV.

The sequence in the VP2/NS junction region was variable and suitable for genogrouping of birnaviruses (Suzuki *et al*., 1997a). At this time, seven genogroups are known in the Aquabirnaviruses (Zhang and Suzuki, 2004), and all MABV have been classified into the same genogroup, which are only of marine origin. Results from Okinawa and Ishigaki samples showed that all sequences belonged to the MABV genogroup, as shown in previous studies (Zhang and Suzuki, 2004). However, a notable finding is that the five-nucleotide deletion occurred at a high frequency. Of the gastropods, bivalves and fish examined, 57.7, 87.0, 100% possessed the same deletion, respectively (Table 3). Transition of C to T at the 1642nd base was reported in monitoring of Japanese shellfish (Suzuki and Nojima, 1999) and an isolated strain from Japanese pearl oyster (Suzuki *et al*., 1998a). Although this transition was not detected in Okinawa samples in this study, another unique mutation was found. The 1630th to 1634th nucleotides were deleted with a high rate of occurrence. This five-nucleotide deletion has never been reported so far. The VP2/NS junction region includes the N terminus of NS protease (Imajoh *et al*., 2007). The NS region acts on virus replication in the host cell, and it is suggested that, in addition, the transition of this region participates in host specificity (Duncan *et al*., 1987; Magyar and Dobos, 1994). Moreover, the presence of the several cleavage sites was proposed in the NS region (Imajoh *et al*., 2007). Some of the cleavage sites were conserved only in the MABV strains, but not in the IPNV strains (Pitit *et al*., 2000; Imajoh *et al*., 2007). The amino acid residue of these cleavage sites suggests that they have an important role in the protease activity of NS. The reason why the replication of the two isolates in this study occurred in spite the deletion of the five nucleotides should be examined in the future. It might be probable that 1) the NS enzyme activity can be compensated by host protease, and 2) another mutation in downstream of the five nucleotides deletion might recover the VP3 structural protein although the structure is changed.

In conclusion, genome detection and virus isolation were applied in this study to examine the distribution and the genetic characteristics of MABV from shellfish and fish in subtropical waters of Okinawa and Ishigaki Islands, which have high water temperatures throughout the year. Results of viral genome detection revealed the presence of MABV in marine organisms in areas with high water temperatures. Knowledge of the distribution of MABV in subtropical waters is essential in understanding the ecology of the virus. Furthermore, the unique five-nucleotide deletion was found with a high rate of occurrence in the MABV genome from shellfish and fish. The function of the deletion is of interest in the evolution of birnaviruses; further study is warranted.

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