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Red sea bream iridovirus infection in marble goby (Bleeker, *Oxyeleotris marmoratus*) in Taiwan

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An outbreak of infectious disease in 30,000 marble gobies (Bleeker, *Oxyeleotris marmoratus*) cultured at a density of 5,000 to 8,000 fish per pond at a fish farm in the central Taiwan was reported to the Nantou County Animal Disease Center and the Central Taiwan Aquatic Disease Center. The mortality rate was approximately 40% (12,000/30,000). In May 2010, clinical signs of sluggish behavior, atypical swimming behavior at the edge of the pond and sudden death were noted among the marble gobies, which ranged from fry (12.7 cm) to juveniles (550 g). Moribund juveniles fish (n=5) were taken for diagnosis. Gross examination demonstrated cutaneous melanosis, local ulcers, and fatty liver. Gill imprint examined under light microscope revealed eggs of *Dactylogyrus crucifer*. Histopathology examination revealed inclusion body-like structures, necrosis, and hypertrophy in the pillar cells of gill, and multifocal proliferation of filament cells in gills. Additionally, lymphoid depletion, macrophages hyperplasia, and hypertrophy with the presence of intracytoplasmic vacuoles in the spleen as well as decreased numbers of melanomacrophages centers and hemocytoblasts in the kidney were observed. The result of polymerase chain reaction (PCR) of our case pathogen yielded a 570 bp fragment, which was consistent with the products of PCR obtained from red sea bream iridovirus (RSIV). The final diagnosis was RSIV infection in marble gobies. Additionally, antimicrobial agent sensitivity test showed no remarkable findings.

Key words: Fish, marble goby, red sea bream iridovirus, Taiwan.

INTRODUCTION

The marble goby (nicknamed Soon Hock), *Oxyeleotris marmorata* (Bleeker), which belongs to Family Eleotridae (Sleepers), Order Perciformes, and Class Actinopterygii (ray-finned fishes), is a tropical freshwater fish found

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throughout East Asia, including Malaysia, Thailand, Indonesia, and Singapore. It can grow to over 1 kg in weight with a maximum body length of 65 cm. It currently fetches a high price in wet markets and restaurants of S$ 28-35/kg and S$ 70-80/kg, respectively. Since marble goby has a high commercial value, it is extensively cultured in Malaysia, Cambodia, Vietnam, and Indonesia (Luong et al., 2005).

Red sea bream iridovirus (RSIV) has been shown to have a wide geographical distribution and host range (Matsuoka et al., 1996; Miyata et al., 1997). Since 1990, large-scale outbreaks of RSIV disease have resulted in high mortalities and severe economic losses in cultured red sea bream and striped beakperch in south-west Japan (Inouye et al., 1992), Korean (Jung and Oh, 2000), and Taiwan (Wang et al., 2003). Outbreaks of fish diseases occurring primarily in the summer are characterized by an enlarged spleen and enlarged basophilic cells in the heart, liver, spleen, kidney, and gill. Icosahedral virus particle size often observed in necrotic cells is 200-240 nm in diameter.

An outbreak of RSIV infection in hybrid tilapia (*Oreochromis niloticus* × *Oreochromis Mossambicus*) occurred at the fish farm from May to June 2009. In May 2010, an apparently similar disease occurred in cultured marble goby at the same fish farm. The results of clinical signs, histopathology, PCR amplification, sequencing, and comparative analysis revealed the pathogen of this infection in marble goby was red sea bream iridovirus. In this study, we report recent outbreaks of RSIV in marble goby (Bleeker, *Oxyeleotris marmorata*) cultured in Taiwan and describe for the first time, RSIV associated with mortality (Huang et al., 2011) and report red sea bream iridovirus infection in marble goby worldwide (Wang et al., 2011).

MATERIALS AND METHODS

Fish

Approximately 30,000 marble gobies were raised on a fish farm at a density of 5,000 to 8,000 fish per pond in the Nantou County, central Taiwan. The ponds contained aerated groundwater (water temperature was maintained at 25 ± 2°C). In this case, moribund juvenile fishes (n=5, 20 cm) were taken for diagnosis and analysis.

Clinical, gross, and histopathologic examination

The clinical, gross, and histopathological examination of ill fish was performed by a clinical doctor of veterinary medicine and fish disease specialist. The marble gobies, ranging from fry (12.7 cm) to juveniles (550 g), showed clinical signs of atypical swimming behavior with no response and sudden death. The mortality rate during this period reached approximately 40% (12,000/30,000). Five of the moribund juvenile fishes were euthanized with Urethane® (Sigma, USA). Samples were collected from the gill, skin, liver, spleen, and kidney of the ill fishes for histopathological examination. All slides were stained with hematoxylin and eosin (HE) (BBC Biochemical, USA) and observed under upright microscope (ECLIPSE 90i, Nikon, Japan).

Polymerase chain reaction detection and sequencing

Polymerase chain reaction (PCR) was performed and the forward and reverse primers were1-F: 5'-CTC AAA CAC TCT GGC TCA TC-3' and 1-R: 5'-GCA CCA ACA CAT CTC CTA TC-3', respectively (Wang et al., 2003). Amplification reactions were performed in a total volume of 25 µL containing 16.5 µL DEPC water, 2 µL dNTP (2.5 µM), 2.5 µL 10× buffer, 1 µL Taq DNA polymerase, 1 µL DNA template obtained and quantified from RSIV-affected gill tissue, and 10 µM of each primer (1 µL). The DNA was quantified before it was used in the PCR reaction. Wild type RSIV isolated from red sea bream was served as positive control. DNA sequences were amplified in a thermal cycler (Gene Amp PCR System 9700; Applied Biosystems, Foster City, CA, USA) using the following program: 94°C for 5 min, followed by 30 cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min, then a final extension period at 4°C. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (2 µL) on 2% agarose gel with healthy nucleic acid staining in 1× TAE buffer. PCR products were cut from 1% low-melting point agarose gel then purified by silica adsorption using a QIAquick gel extraction kit (Qiagen). Sequence analysis was then performed using primer mc-1 and an ABI cycle sequencer dRhodamine Big Dye kit. The reaction products were analyzed using an ABI model 377 automated sequencer. Nucleotide sequences were compared to the known sequences published in the GenBank using the Blast program. Nucleotides were aligned using the Genetyx program.

RESULTS

Clinical signs, gross and histopathologic findings of ill marble goby

Clinical examination of the ill marble goby showed clinical signs of atypical swimming behavior with no respond and sudden death. The gross findings revealed cutaneous melanosis, local ulcers, and fatty liver (Figure 1). Gill imprint examined under light microscope revealed eggs of *Dactylogyrus crucifer* (data not shown). The histopathological examination revealed inclusion body-like structures, necrosis, and hypertrophy in the pillar cells of gill and multifocal proliferation of filament cells in gills (Figure 2). Lymphoid depletion, macrophages hyperplasia, and hypertrophy with the presence of intracytoplasmic vacuoles in the spleen as well as a decrease in the number of melanomacrophages centers and hemocytoblasts in the kidney were noted (Figure 2).

Sequencing and comparison of PCR amplifications of viral DNA

The results of PCR showed the specific gene products 570 bp obtained from PCR in our case pathogen (Figure 3) were 100% consistent with the PCR gene products of RSIV (Access number in GenBank: AF506370.1) (data not shown). These findings confirmed that our case pathogen was RSIV.

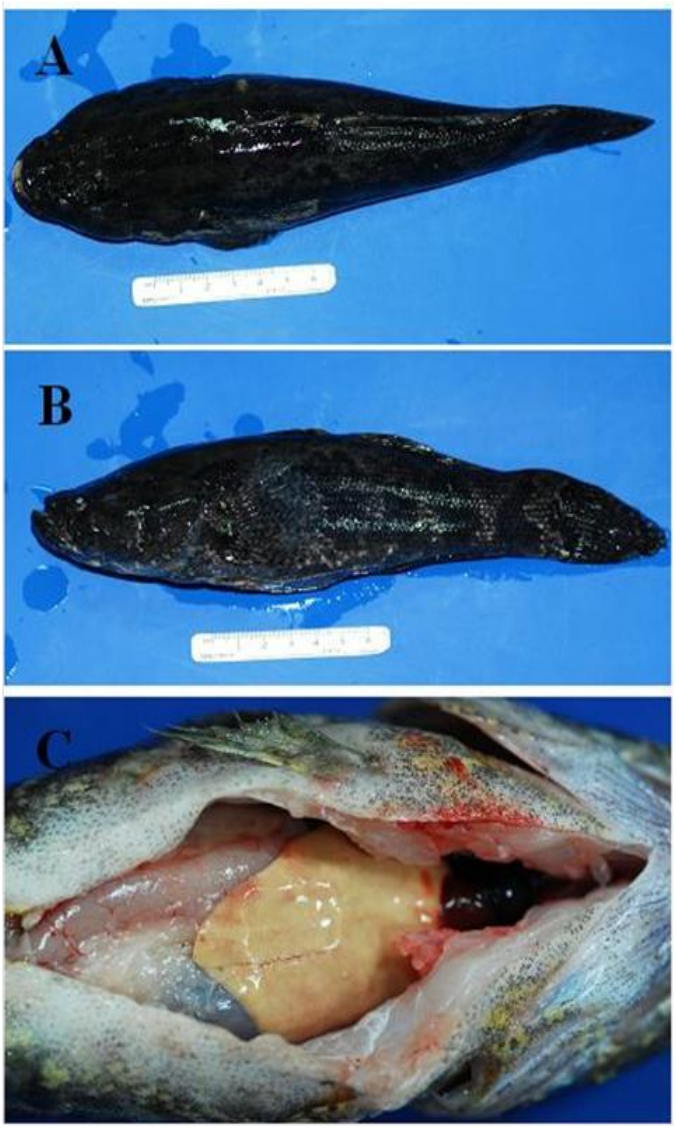

Figure 1. Gross examination of diseased marble goby. (A-B) The color change, cutaneous melanosis, and local ulcers of the diseased fish dorsum and side were found. (C) The ill fishes had fatty liver.

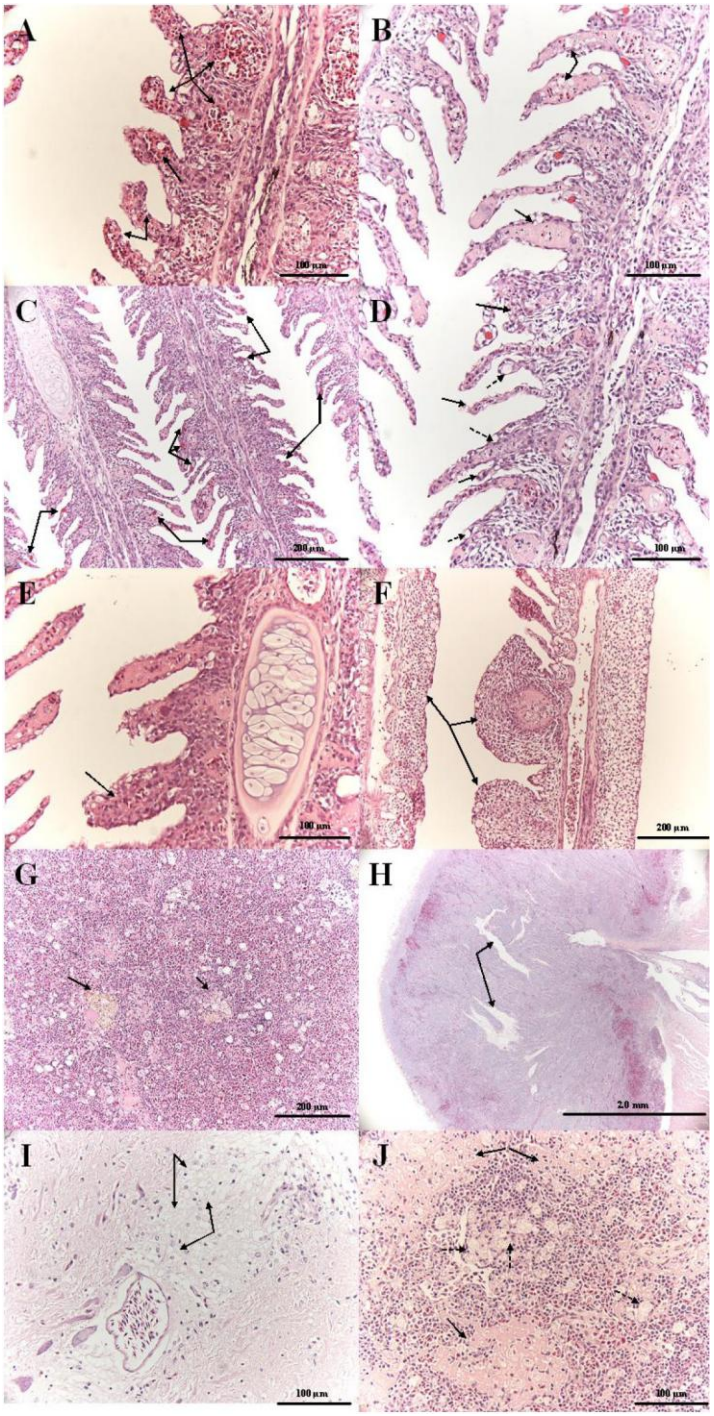

Figure 2. Histopathologic examination of ill marble goby. (A) Congestion in secondary gill plate (arrows). (B) Vessel thrombosis and inflammation in vessel wall (arrows). (C) Inclusion body-like structures in the pillar cells of gill (arrows). (D) Necrosis (solid arrows) and hypertrophy (dotted arrows) in the pillar cells of gill. (E) Multifocal proliferation of filament cells in gill (arrow). (F) Proliferation of the pillar cells (arrows). (G) Decrease in the number of melanomacrophages centers and hemocytoblasts in the kidney (arrows). (H) Intimal hyperplasia in arterial ball. (I) Vacuolation in brain medulla and brain stem region. (J) Lymphoid depletion (solid arrows), macrophages hyperplasia (dotted arrows), and hypertrophy with the presence of intracytoplasmic vacuoles (dotted arrows) in the spleen.

DISCUSSION

Based on the results from clinical signs of ill fish, histopathologic examination, and gene amplification, sequencing, and comparison of PCR, the causative agent of cultured marble goby in Taiwan was demonstrated to be RSIV. Outbreaks of RSIV have caused mass mortalities and severe economic losses. Epizootics of iridovirus disease in marine fish have been reported in Taiwan, Singapore, Japan, Korea, Thailand, and Hong Kong (Inouye et al., 1992; Chua et al., 1994; Miyata et al., 1997; Chou et al., 1998; Jung and Oh, 2000). High mortality rates with RSIV infection were found in cultured marine fish such as red sea bream, grouper, yellowtail,

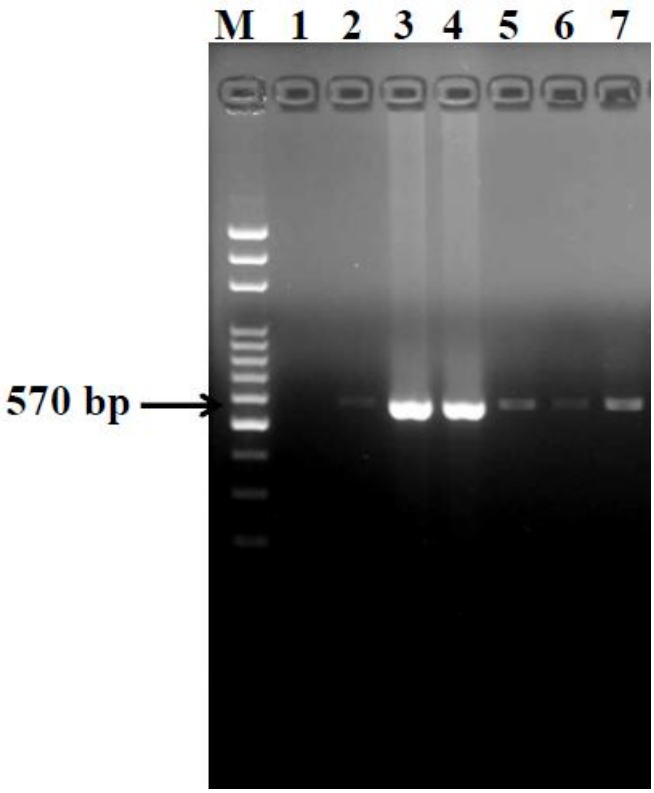

Figure 3. PCR amplification showed specific gene products. The 570 bp amplicon was obtained from PCR. Lane 1: Healthy fish gill tissue of marble gobies served as the negative control; lane 2: RSIV served as positive control (wild type RSIV isolated from red sea bream); lanes 3-7: gills of ill marble gobies; M: DNA molecular marker (100 bp ladder).

sea bass, and Japanese parrotfish (Danayadol et al., 1997; Nakajima et al., 1998a). This is the first case report of RSIV infection in cultured marble goby in Taiwan. Since marble goby is high commercial value and their gross and histopathology information under RSIV infection were not enough in Taiwan, it is recently and extensively cultured in Taiwan. In this study, we focused on the gross and histopathological characteristics of RSIVD in the marble goby. We hope these useful information will be provided to the feeder and researchers.

RSIV outbreaks in Japan usually occur during the warmer summer months; therefore, the occurrence of iridovirus infection in red sea bream appears to be correlated with fluctuations in temperature (Inouye et al., 1992; June and Oh, 2000; Wang et al., 2003). In Taiwan, juvenile red sea bream are transported to Penghu Island from hatcheries in March. Epizootic mortalities were found to increase with rises in water temperature during the summer (Wang et al., 2003). In our case, an outbreak of disease with RSIV infection occurred in May and the water temperature of the culture ponds was about 25 ± 2°C. Thus, the water temperature and season were optimal for RSIV outbreaks. Additionally, Huang et al. (2011) also reported that rock bream iridovirus (RBIV/Tp/45/08) even invaded Taiwanese fish farms in 2008 and its genotype was closed to Korean flounder iridovirus (FLIV) and turbot reddish body iridovirus (TRBIV). The strain originated from diseased rock bream that was imported as larvae from Korea. Thus, the route and source of fish import may be considered the important factors in this case. Genetic analysis of fish iridoviruses isolated in Taiwan during 2001-2009 showed that iridoviruses isolated from Taiwan aquaculture fishes could be classified into two genotypes. One type is the viruses closely related to the genus Ranavirus before 2005 and another is similar to the genus Megalocytivirus after 2005. These date explored that no RSIV (megalocytivirus genotype I) has been found in Japan or Korea since 2005. At present, all megalocytivirus detected in outbreraks of Asian countries were rock bream iridovirus (RBIV) (megalocytivirus genotype II). This change will be more worthwhile to analyze the possible sources (or routes) of spreading/transmission of RSIV (megalocytiviruses genotype I) in marble goby with the monitoring data in environment of Taiwan (Huang et al., 2011). Recently, ISKNV and RSIV genotype I and II infected freshwater fishes were undertaken in China. Based on these findings, it is possible that this freshwater fish species may be a “mixing vessel” for megalocytiviruses and underlies the danger of identifying a virus based only on the species of fish infected. In addition, some cases were reported that the distribution of ISKNV remains restricted to Southeast Asia, Taiwan and China and has not yet been found in Japan or South Korea. The distribution of TRBIV is restricted to areas around the Yellow Sea in East Asia and has not yet been found in Southeast Asia. Hong Kong, a base for fish seedling export, is a hotspot for megalocytiviruses. Presence of ISKNV and RSIV genotypes I and II has been confirmed in Hong Kong and it is possible that all Asian megalocytiviruses, except TRBIV, spread from this area (Kurita and Nakajima, 2012).

In clinical, RSIV infection, Murray cod iridovirus, and lymphocystitis disease should be differential diagnosis. We first excluded lymphocystitis disease was the major reason of outbreak in this case according to clinical signs and mortality of fish and histopathology examination. Furthermore, the results of PCR only showed 93% consistent with the PCR gene products of Murray cod iridovirus (Access number in GenBank: AY936206.1) (data not shown). Therefore, many evidences like the same pathogen RSIV infection in the same fish farm in the previous year, inclusion body-like structures in the gill tissues were found under the histopathological examination, and results of PCR, sequencing, and comparison were demonstrated that RSIV was a unique pathogen in this case.

In Japan, RSIV led to losses of up to 20-60% in fingerling

and market-sized red sea bream (Nakajima et al., 1998b), whereas only juvenile fish of red sea bream were affected. The cumulative mortality of this disease reached 50-90% in Taiwan (Wang et al., 2003). It may be that survivors infected in their first year had acquired protective immunity, and so were refractory to further serious disease (Wang et al., 2003). In our case, the same fish farm reported an outbreak of RSIV in marble gobies in the previous year. Therefore, the mortality rate was about 40%, predominantly in fry and juveniles. There were no suspicious signs of disease in adult fish, which suggests that survivors infected in the previous year may have acquired protective immunity against RSIV. Additionally, the other possibility was considered that marble goby are not assymptomatic carriers of RSIV.

At present, there are three viral species exist in the genus *Megalocytivirus* according to the genetic analysis of the major capsid protein and ATPase genes. One is RSIV, the first isolated from the cultured red sea bream (*Pagrus major*) in Japan in 1990. Others is represented by infectious spleen and kidney necrosis virus (ISKNV) isolated from freshwater Chinese perch (*Siniperca chuatsi*) and turbot reddish body iridovirus (TRBIV) isolated from turbot (*Scophthalmus maximus*) (Kurita and Nakajima, 2012). Histopathological signs of ill fishes with megalocytivirus infections were showed in many articles (Wang et al., 2003; Shi et al., 2004; Wang et al., 2008). In RSIV-infected fishes, spleen and kidney were the most affected organs. Therefore, histopathological observations were consistent with the anaemia and splenomegaly observed in ill fish. Enlarged cells in the spleen, heart, kidney, liver and gills of affected fish characterize red sea bream iridovirus disease (RSIVD) (Wang et al., 2003). Under the gross exanimation, the prominent external features of ISKNV infection are petechial hemorrhages in the gill cover, lower jaw, eye, base of dorsal and ventral fins, caudal fin, and abdomen. The histopathology exanimation of ISKNV infection is characterized by cell hypertrophy in the spleen, kidney, cranial connective tissue, and endocardium. Infected cells are enlarged with large numbers of icosahedral viral particles (150 nm) present in the cytoplasm (Wang et al., 2008). Many enlarged cells distributed in various tissues of diseased fish, especially in the TRBIV affected spleen and kidney. The enlarged cells (15-20 μm in diameter) with a basophilic cytoplasm were presented. Spleen displayed disruption of ellipsoid sheaths with degeneration of associated cells and the degenerating splenic pulp in ill fishes. In kidney, the endothelial cells and podocytes in glomeruli were enlarged and homogeneous (Shi et al., 2004). In our study, histopathologic examination revealed inclusion body in the pillar cells of gill, and hypertrophy was found in the cells of gill and spleen, which are also characteristics of RSIV disease. Taken together, the most obvious characteristics of the megalocytivirus infection were the widespread necrosis of tissues and enlargement of cells. The spleen and kidney were the most affected organs in RSIV infected fish (Miyata et al., 1997; Kurita et al., 1998; Oshima et al., 1998). Haematopoietic tissue is located in the stroma of the spleen and the interstitium of the kidney in teleosts. Therefore, histopathological observations were consistent with the anaemia and splenomegaly observed in RSIV-infected fish. Enlarged cells were found in various tissues including spleen, kidney, liver, heart, and gill. Development of enlarged cells in the spleen, heart, kidney, liver, and gills of affected fish are characteristic of megalocytivirus infection (Nakajima and Sorimachi, 1994). However, it is difficult to distinguish what kind of subtypes of megalocytivirus infection just according to histopathology examination. Hence, the enlarged cells were basophilic with H&E and stained blue with Giemsa. Giemsa stain imprints of the spleen have been commonly used for the rapid diagnosis of RSIV-infected fish in the field in Japan and can also be recommended as a simple diagnostic tool in Taiwan (Wang et al., 2003). Currently, indirect immunofluorescence tests employing recently generated monoclonal antibodies and PCR assays are currently used in the rapid diagnosis of megalocytivirus infection (Kurita and Nakajima, 2012).

A number of primer sets specific for RSIV have been designed (Miyata et al., 1997; Kurita et al., 1998; Oshima et al., 1998). In this study, three RSIV-specific primer sets were used in PCR to identify suspected viral nucleic acid in the gill of infected fish. A PCR product of 570 bp was amplified using the primer set with a DNA template obtained from affected gill tissue. Conversely, no product was found when using the gill tissues of healthy fish as a template. Many important genes were used to analyze the genetic relationships in members of the family *Iridoviridae* included major capsid protein, adenosine triphosphatase, DNA polymerase and methyltransferase genes (Whittington et al., 2010; Huang et al., 2011). Miyata et al (1997) reported that RSIV isolates from Japan and Thailand were genetically similar, which suggests the widespread distribution of RSIV with a single origin and a wide host range. Wang et al (2003) reported that Taiwan RSIV was genetically similar and close genetic affinities with Japan RSIV. Gene sequencing of our case pathogen compared with that of Japanese RSIV was 100% identical. These results indicate that the viral agent in cultured marble gobies in the current study is closely related to the RSIV found in Japan and also suggests that RSIV may have been introduced to Taiwan.

Taken together, the clinical isolate identified in our analysis provides the first evidence that RSIV infection can cause a fatal epidemic disease in cultured marble goby. Based on our findings, we recommended that the fish farm owner adopt the following strategies: (1) control the water temperature to avoid large fluctuations as far as possible, (2) remove ill fish immediately, (3) reduce fish stock density, (4) inspect the fry source, and (5) avoid

introducing fish from one pond into another to prevent cross infection.

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