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Studies on epizootic iridovirus infection among red sea bream, *Pagrus major* (Temminck & Schlegel), cultured in Taiwan

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Abstract

Since 1993, an epizootic viral disease has occurred in net-cage cultured red sea bream, *Pagrus major* (Temminck & Schlegel), in Peng-hu Island located on the south-western coast of Taiwan. The diseased fish exhibited abnormal swimming and were lethargic, but few visible external signs were observed. The cumulative mortality because of the disease sometimes reached 50–90% over 2 months. Histopathogical studies of the affected fish showed enlarged basophilic cells in the gill, kidney, heart, liver and spleen. These necrotic cells were Feulgen-positive and stained blue using Giemsa. Transmission electron microscopy revealed icosahedral virions in the cytoplasm of the necrotic cells. The viral particles consisted of a central nucleocapsid (75–80 nm) and envelope, and were 120–150 nm in diameter. These results suggest that the virus belongs to the Iridoviridae. Using polymerase chain reaction (PCR), approximately 570 bp fragments were produced from the viral DNA using as a template 1-F and 1-R primers derived from red seabream iridovirus (RSIV) from red sea bream in Japan. Similar results were also obtained using nested-PCR with different primer sets (1-F, 2-R and 2-F, 1-R). Although the size and some features of epizootics of this virus differed from RSIV in Japan, it shows close genetic affinities with the latter and it is suggested that RSIV has been introduced to Taiwan.

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Introduction

Mass mortalities have occurred in net-cage cultured red sea bream, *Pagrus major* (Temminck & Schlegel), at Peng-hu Island, Taiwan from 1993. The affected fish displayed abnormal swimming and lethargy, but no visible external signs were seen. The cumulative mortality of fish reached 50–90% within 2 months and resulted in significant economic losses.

Since 1990, outbreaks of red sea bream iridovirus disease (RSIVD) have resulted in high mortalities in cultured red sea bream in south-west Japan, primarily in the summer. The affected fish were characterized by an enlarged spleen and the presence of enlarged basophilic cells in the spleen, gill, kidney, heart and liver. Icosahedral virus particles, 200–240 nm in diameter, were observed in the necrotic cells, and tentatively designated as red sea bream iridovirus (RSIV) (Inouye, Yamano, Maeno, Nakajima, Matsuoko, Wada & Sorimachi 1992). The RSIV has been shown to have a wide geographical distribution and host range (Matsuoka, Inouye & Nakajima 1996; Miyata, Matuno, Jung, Danayadol & Miyazaki 1997). A number of diagnostic methods have been developed for detecting RSIV, including cell culture (Nakajima & Sorimachi 1994), immunofluorescence (Nakajima, Maeno, Yokoyama, Kaji & Manabe 1998b) and polymerase chain reaction (PCR) (Oshima, Hata, Hirasawa, Ohtaka, Hirono, Aoki & Yamashita 1998). A similar disease is known to occur among

cultured grouper, *Epinephelus* spp., in Taiwan, and the causative viruses have been isolated and characterized (Chou, Hsu & Peng 1998). The present study provides histopathological and electron microscope evidence of iridovirus infection of red sea bream cultured in Taiwan. In addition, the genetic relationships between isolates of RSIV from Taiwan and Japan was investigated using PCR.

Materials and methods

Fish

Diseased red sea bream (7–10 cm total length) were collected from Peng-hu Island on the south-western coast of Taiwan. Healthy red sea bream about 2 years old were sampled from the same farms. These fish were submitted to histological study, transmission electron microscopy (TEM) and PCR as described below.

Histology

For histological studies, the spleen, kidney, liver, heart and gill were taken from diseased fish. The tissue samples were fixed in 10% neutral buffered formalin and processed for paraffin wax sections. 5–7 μm sections were stained using haematoxylin and eosin (H&E), Giemsa and Feulgen stains.

Electron microscopy

Pieces of spleen, gill, heart and kidney from affected fish were fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffered phosphate (pH 7.2) at 4 °C for 1 h. After several rinses with buffer solution, the samples were post-fixed with 1% OsO_4 for 1 h. Subsequently, the tissues were dehydrated in an ethanol series and embedded in Spurr's resin. Ultrathin sections were cut with a Sorvall MT-5000 ultratome (Sorvall, DuPont, Newtown, CT, USA), stained with uranyl acetate and lead citrate, and observed with a TEM (HITACHI H-600, Hitachi, Japan) at 75 kV.

DNA extraction

Spleen, kidney, liver, heart and gill were removed from diseased red sea bream and homogenized in lysis buffer (50 mM Tris–HCl, pH 8; 20 mM NaCl, 2% SDS, 10 mM EDTA, pH 8; proteinase K 100 μg mL^{-1}) for 3 h at 56 °C. The DNA was extracted by the phenol/chloroform method as described by Sambrook, Fritsch & Maniatis (1989).

PCR amplification and analysis of products

The oligonucleotide primers (1-F 5′-CTCAAACA CTCTGGCTCATC-3′ and 1-R 5′ GCACCAA CACATCTCCTATC-3′) used in this experiment were derived from the DNA sequence of the 959-bp *Pst* I fragment of an iridovirus from red sea bream (Kurita, Nakajima, Hirono & Aoki 1998). Amplification products of about 570 bp were generated. The PCR amplification was performed under conditions previously described by Kurita *et al.* (1998). Briefly, the amplification procedure was carried out in a 50 μL reaction mixture containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 100 pmol of each primer, 1 unit of *Taq* DNA polymerase and 1 μg DNA template. A drop of mineral oil was added to cover the mixture. The mixture was incubated in a PTC-100 DNA thermal cycler (MJ Research Inc., USA) at 94 °C for 5 min, and then for 30 cycles (94 °C for 30 sec, 58 °C for 1 min, and 72 °C for 1 min) plus a final 5-min extension at 72 °C. The PCR products were analysed in 1.5% agarose gels containing ethidium bromide at a final concentration of 0.5 μg mL^{-1} and visualized under a UV transilluminator.

Nested-PCR amplification

Nested-PCR amplification was performed using different primer sets 1-F, 2R (5′-GCGTTAAAG TAGTGAGGGCA-3′) and 2-F, 1R (5′-TAC AACATGCTCCGCCAAGA-3′) derived from the same 959 bp *Pst* I fragment of RSIV (Kurita *et al.* 1998). The first PCR amplifications were carried out with the primer set 1-F and 2-R, and then the second amplifications were performed with the other primer set 2-F and 1-R. The fragment produced was about 850 bp and diluted 1/1000 as a DNA template for the second amplifications. The reaction conditions and analysis were as described above.

Results

Outbreaks of iridovirus disease occurred from April to June in Taiwan, when water temperature was elevated from 18–20 °C to 25–27 °C. Epizootic disease only occurred in juvenile fish, and no

mortality was observed in 1–2-year-old fish cultured in the same farms. The diseased fish displayed erratic swimming and lethargy, but no visible external lesions were detected. On dissection, an enlarged spleen was occasionally observed. At the beginning of an outbreak, only 3–5% of total fish were affected per day, but the cumulative mortality could reach 50–90% over the following 2 months.

Enlarged cells were found in various tissues including spleen, kidney, liver, heart and gill (Figs 1–4). The enlarged cells were basophilic with H & E and stained blue with Giemsa. The Feulgen reaction was positive, suggesting the presence of DNA in the affected cells. The spleen was the most severely affected tissue and displayed spongiosis and disruption of ellipsoid sheaths with degeneration of associated cells (Fig. 3). Sometimes the splenic pulp was replaced with erythrocytes. The tubular epithelium of the kidney occasionally showed degeneration and was detached from the interstitial tissues. The reticuloendothelial elements consistently showed evidence of necrosis (Fig. 2).

Transmission electron microscopy revealed icosahedral-shaped enveloped virions in the cytoplasm of necrotic cells. Free viral particles were sometimes also found in intercellular spaces (Fig. 5). The viral particles measured approximately 120–130 nm between opposite sides and 140–150 nm between opposite vertices. Each virion consisted of an electron translucent zone and a central electron dense core, measuring 75–80 nm in diameter (Fig. 8). The virion envelopes were clearly trilaminar which is a regular characteristic of iridoviruses. Vesicular structures were commonly abundant in the cytoplasm of necrotic cells, with complete virions aggregated within the vesicles (Figs 6 & 7). Electron-dense bodies were observed in degenerated cells and a large amount of fibrillar material was also present. Virus was detected in all spleen, kidney, gill, heart and liver samples from diseased fish, and the highest numbers of virus particles were seen in the spleen. In some degenerated cells, envelope-like materials and empty envelopes were detected in the cytoplasm (Fig. 9). Budding of virus into vesicles was also found, suggesting that viral assembly might occur in the cytoplasm.

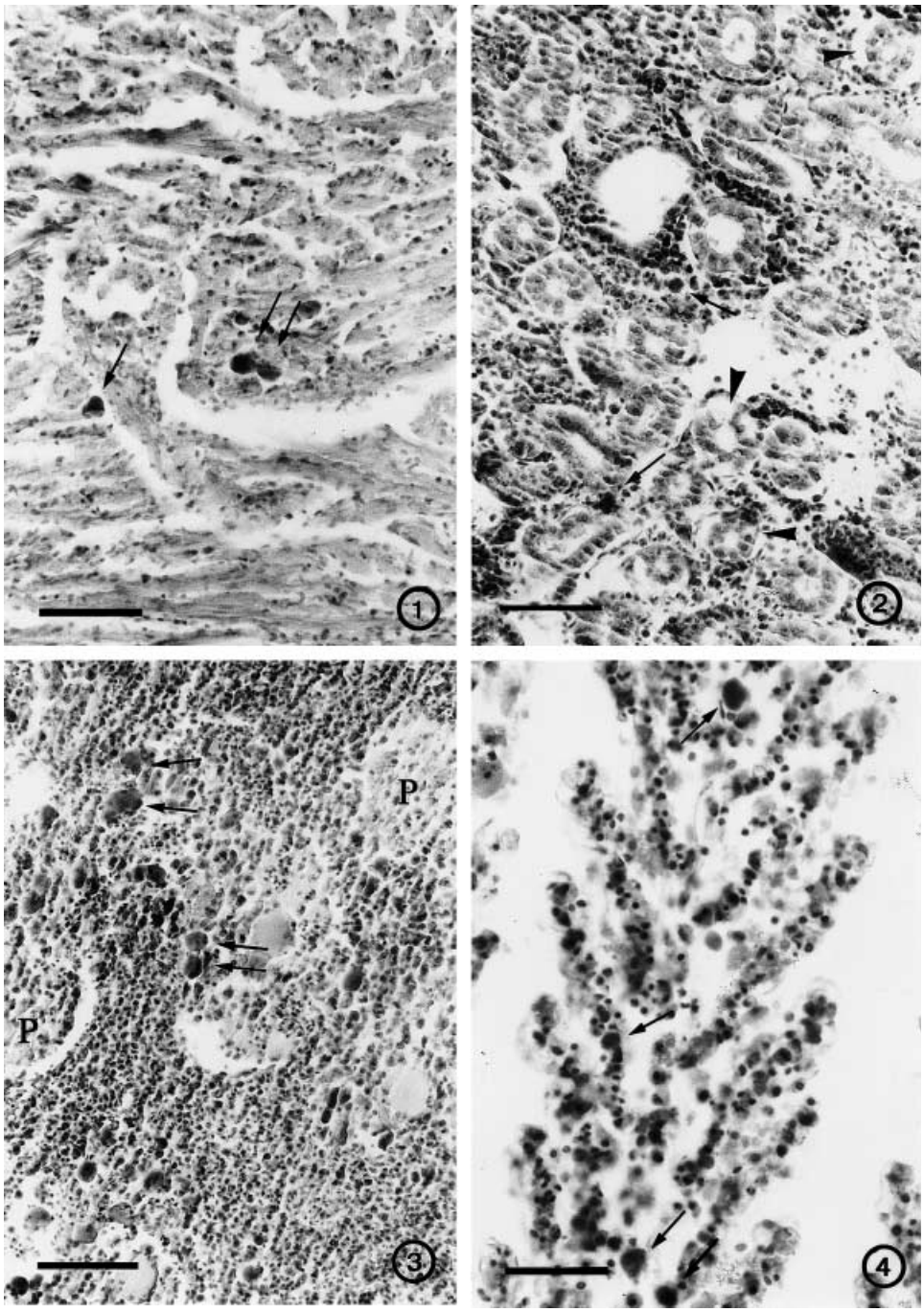
The results of PCR using the 1-F and 1-R primer set are shown in Fig. 10. The PCR amplification products corresponding to 570 bp were amplified from the spleen, kidney, liver, heart and gill of the infected fish. However, these PCR products were absent when purified DNA from the same tissues of healthy red sea bream was used as the template. Figure 11 shows the results of nested-PCR amplification using different primer sets derived from the same 959 bp *Pst* I fragment of RSIV. The first PCR amplifications produced an 850-bp fragment (lane 1), which was similar to the results obtained using RSIV DNA as the template. Using the internal primer set (2-F and 1-R), a major band with a size of approximately 300 bp was obtained.

Discussion

Based on the results from light microscopy, electron microscopy and PCR, it was concluded that the causative agent of mass mortalities of cultured red sea bream in Taiwan was a new isolate of RSIV. Epizootics of iridovirus disease in marine fish have been reported in several Asian countries including Taiwan, Singapore, Japan, Korea, Thailand and Hong Kong (Chou *et al.* 1998; Chua, Ng, Ng, Loo & Wee 1994; Inouye *et al.* 1992; Jung & Oh 2000; Kasornchandra & Khongpradit 1997; Miyata *et al.* 1997). Outbreaks of this disease have caused mass mortality among cultured marine fish including red sea bream, *P. major*, grouper, *Epinephelus malabaricus* (Bloch & Schneider), and *E. tauvina* (Forskal), yellowtail, *Seriola quinqueradiata* (Temminck & Schlegel), sea bass, *Lateolabrax* sp., and Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel) (Danayadol, Direkbusarakom, Boonyaratpalin, Miyazaki & Miyata 1997; Nakajima, Inouye & Sorimachi 1998a). The present study is the first report of histological and electron microscopical evidence of RSIV infection in net-cage cultured red sea bream in Taiwan.

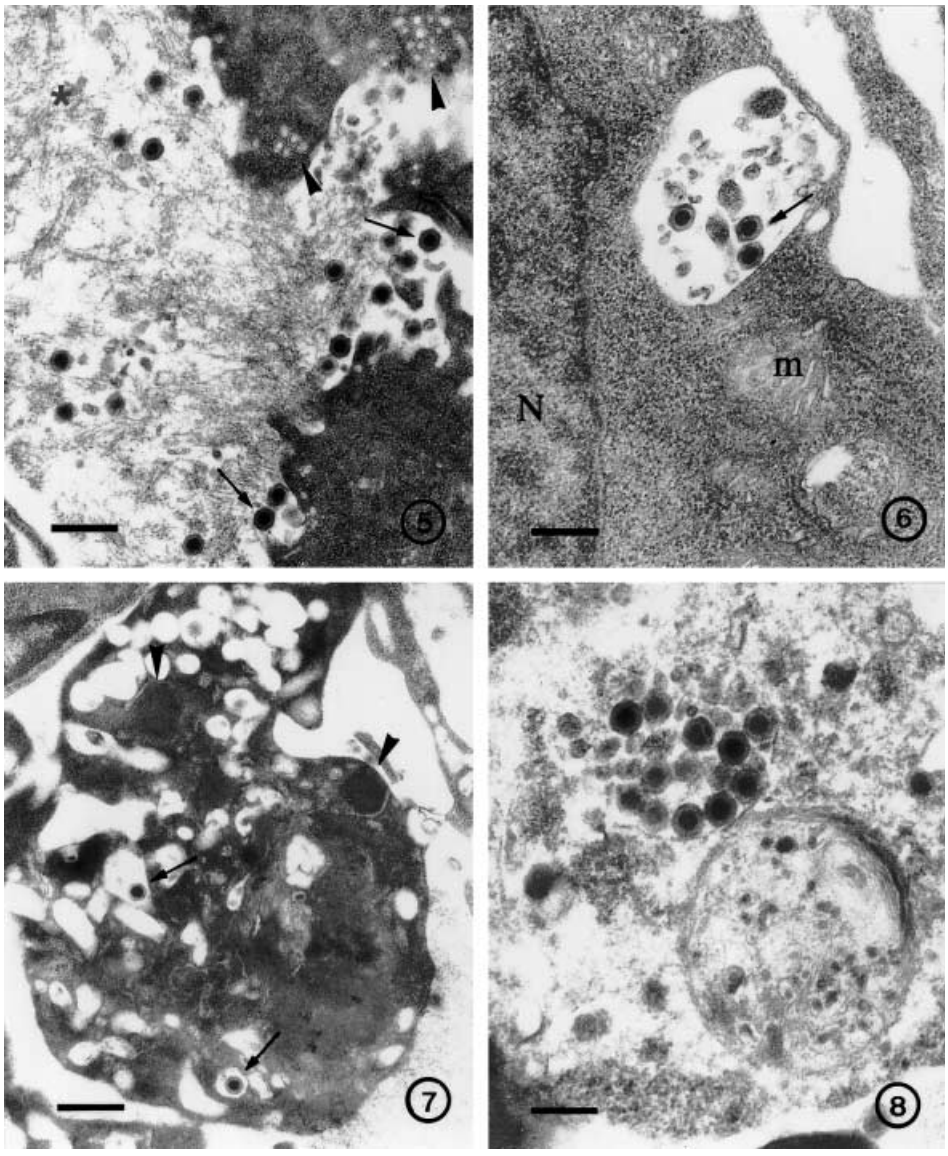
The occurrence of iridovirus infection in red sea bream appeared to be correlated with fluctuations in temperature. In March, juvenile red sea bream were transported to Peng-hu Island from hatcheries in Taiwan. The water temperature was elevated when the epizootic mortalities occurred. The RSIV outbreaks in Japan occurred at summer temperatures. However, 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 were affected in Taiwan. It may be that survivors infected in their first year had acquired protective immunity, and so were refractory to further serious disease.

The spleen and kidney were the most affected organs in infected fish. Haematopoietic tissue is located in the stroma of the spleen and the



Figures 1–4 Light micrographs of tissues from red sea bream iridovirus (RSIV)-infected fish cultured in Taiwan. (1) Heart showing the enlarged cells (arrows) in the ventricle (H&E, bar = 50 μm). (2) Kidney. Arrows indicate enlarged cells, the tubular epithelium displaying degeneration and detachment from the interstitial tissue (arrowheads) (H&E, bar = 50 μm). (3) Spleen. The splenic pulp (P) and ellipsoids show necrosis and degeneration; arrows indicate the enlarged cells (H&E, bar = 50 μm). (4) Gill. Arrows indicate RSIV-infected cells (Giemsa, bar = 50 μm).

interstitium of the kidney in teleosts. Therefore, histopathological observations were consistent with the anaemia and splenomegaly observed in RSIV-infected fish. Development of enlarged cells in the spleen, heart, kidney, liver and gills of affected fish characterize RSIVD. Giemsa stain imprints of the



Figures 5–8 Electron micrographs of spleen cells from RSIV-infected red sea bream. (5) Free virus particles (arrows) in the intercellular space. Arrowheads indicate the vesicles; *fibrillar material (bar = 400 nm). (6) Mature virion (arrow) within a vesicle. Virus particles are surrounded by a trilaminar envelope. N: nucleus; m: mitochondria (bar = 333 nm). (7) Large numbers of vesicles in a necrotic cell, with virus particles (arrows) located within the vesicles. Arrowheads indicate the electron-dense body (bar = 500 nm). (8) Aggregation of virus in the cytoplasm (bar = 250 nm).

spleen have been commonly used for the rapid diagnosis of RSIV-infected fish in the field in Japan (Nakajima *et al.* 1994) and can also be recommended as a simple diagnostic tool in Taiwan.

Icosahedral virus particles were detected in the cytoplasm of necrotic cells. The virus found in Taiwan was 140–150 nm in diameter, and was thus smaller than Taiwan grouper iridovirus (TGIV) (Chou *et al.* 1998) and RSIV from Japan. They were similar to iridovirus found in cultured grouper suffering from 'sleepy grouper disease' in Singapore (Chua *et al.* 1994). In some affected cells, large numbers of intracytoplasmic vesicles were found. This vacuolation was similar to that seen in white sturgeon iridovirus (WSIV) at early infection stages of fish cell lines (Watson, Groff & Hedrick 1998), and suggests it might be indicative of early infection and synthesis of the virus. The different stages of virus assembly observed in the cytoplasm here are similar to WSIV replication in sturgeon cell lines.

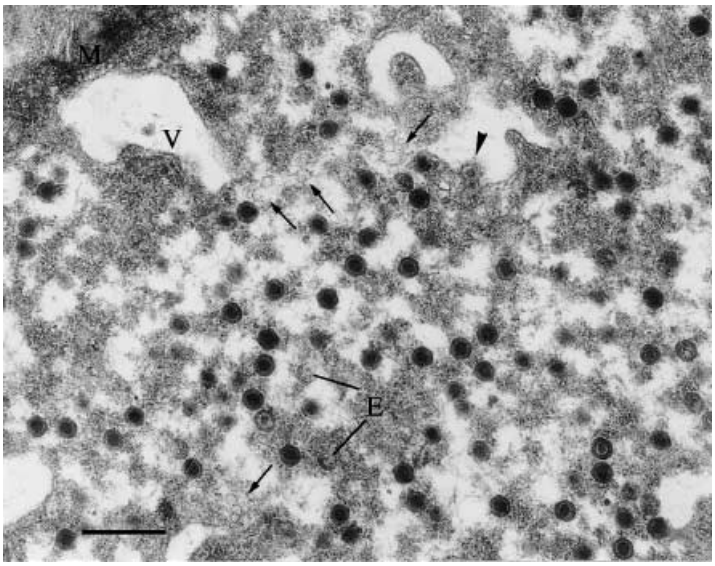


Figure 9 Transmission electron micrograph of RSIV showing viral envelope-like materials (arrows), and a virion budding into a vesicle in the cytoplasm (arrowhead). E: empty envelope, M: mitochondrion, V: vesicle (bar = 500 nm).

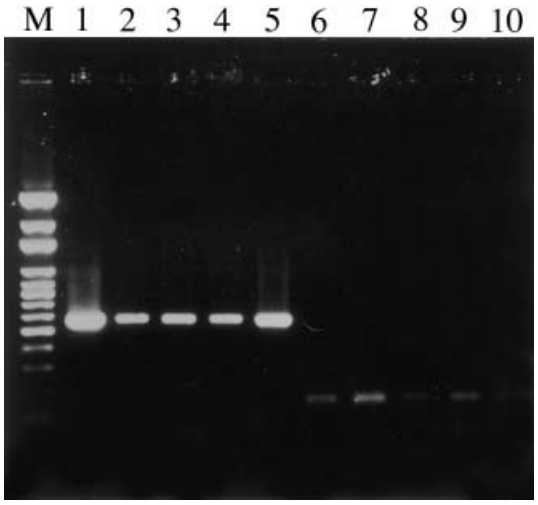


Figure 10 The PCR products from RSIV-infected red sea bream cultured in Taiwan using a specific primer set (1-F and 1-R). The DNA isolated from various tissues of diseased fish (lanes 1–5) and healthy fish (lanes 6–10). Lanes 1 and 6 spleen; lanes 2 and 7 heart; lanes 3 and 8 liver; lanes 4 and 9 kidney; lanes 5 and 10 gill. M: DNA molecular marker (100-bp ladder).

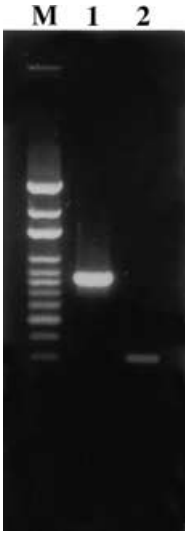


Figure 11 Products of a nested-PCR amplification using primer sets specific for genomic DNA of RSIV. The DNA of RSIV-infected spleen was the template for PCR amplification using the following primer pairs. Lane 1, 1-F and 2-R; lane 2, 2-F and 1-R. M: DNA molecular marker (100-bp ladder).

These results suggests that the virus from red sea bream is a member of the Iridoviridae.

A number of primer sets specific for RSIV have been designed, based on the ATPase gene, DNA polymerase gene, ribonucleotide reductase small subunit and *Pst* I restriction fragments of virus genomic DNA (Miyata *et al.* 1997; Kurita *et al.* 1998; Oshima *et al.* 1998). In this study, three RSIV specific primer sets, 1-F 1-R, 1-F 2-R and 2-F 1-R, were used in a PCR designed to identify suspected viral nucleic acid in the tissues of infected fish. A PCR product of about 570 bp was amplified using the 1-F and 1-R primer set with DNA template obtained from affected tissues. Alternatively, no product was found when using the tissues of healthy fish as a template. In a nested-PCR method, similar results were obtained using different primer sets (1-F 2-R and 2-F 1-R). In addition,

these results were confirmed with RSIV from Taiwan using a primer set (V1 and V5) designed by Oshima *et al.* (1998) (data not shown). These results indicate that the viral agent in the cultured red sea bream in Taiwan is closely related to the RSIV found in Japan. Miyata *et al.* (1997) demonstrated using PCR amplification that RSIV from Japan and an iridovirus from Thailand were genetically similar. This suggests the widespread distribution of RSIV of a single origin and a wide host range, and indicates that RSIV has been introduced to Taiwan. According to Kurita *et al.* (1998), no PCR products were produced using frog virus 3 (FV3) or fish lymphocytis disease virus (FLDV) as the template with the above primer sets. As FV3 and FLDV also belong to the Iridoviridae, it would be worthwhile to study further the relationship between RSIV isolates and other fish iridoviruses.

Although RSIV from Taiwan was detected by PCR using specific primers, the virus was not isolated using established fish cell lines in our laboratory. This is in accordance with similar results in Japan. Chou *et al.* (1998) also reported the instability of TGIV in the KRE cell line as a barrier to the purification of the virus. In order to produce large amounts of viral material for further studies, including characterization of the viruses, and to assist in the development of improved diagnostic procedures, a cell culture system for the *in vitro* multiplication of the RSIV from Taiwan is urgently needed. In addition, a serological identification test for the early detection of virus carriers among broodstock is needed.

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