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Development of a cell line from the gill of koi and molecular characterization of KHV isolated in Taiwan

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A new cell line (KoG) was developed from the gill of koi, *Cyprinus carpio.* KoG cells were composed of epithelial-like cells and fibroblast-like cells. The cells were maintained well at 25°C in Leibovitz L15 medium supplemented with 10% fetal calf serum. Chromosome analysis showed that the KoG cells had 43 diploid chromosomes at passage 40. The KoG cells were used to test the susceptibility of a koi herpesvirus isolate using a Taiwanese koi herpesvirus isolate (strain TW BL98). The KHV-infected KoG cells revealed vacuolation in the cytoplasm and marginal hyperchromation in the nucleus, which was confirmed by an indirect immunofluorescence assay and transmission electron microscopy. A multistep growth curve showed the virus reaching the highest titer of $10^{6.2}$ $TCID_{50}$/ml at 5 days post inoculation. Sequence analysis targeting the alleles of the two molecular markers indicated that the Taiwanese isolate displayed the $I^{++}II^{+}$ allele and of the Asian lineage, which was identical to a Japanese strain (Asian genotype variant 1). Collectively, our results demonstrate that the KoG cells may provide a tool for the replication of KHV and the Taiwanese isolate belongs to a typical Asian genotype.

Key words: Taiwan, KoG cell line, koi herpesvirus, phylogenetic analysis.

INTRODUCTION

Koi Herpesvirus (KHV), recently described as cyprinid herpesvirus-3 (CyHV-3), is known to cause mass mortality in juvenile and adult carp. KHV was first reported in Israel and the United States in 1998 (Hedrick et al., 2000); it then spread rapidly throughout fish trade and related exhibitions worldwide (OIE manual, 2009). In recent years, studies on KHV have received much attention due to its rapid transmission and high mortality, which have resulted in severe financial losses in the koi and carp culture industries. Regarding the control of the disease, many cell lines have been developed to investigate the interactions between the virus and the host *in vitro* (Hedrick et al., 2000; Neukirch and Kunz, 2001; Pikarsky et al., 2004; Davidovich et al., 2007; Dong et al., 2011; Sunarto et al., 2011). However, the difficulty in the isolation of KHV was noted in previous studies, which may be due to unstable features of the virions in the infected tissues during storage and low sensitivity of the cell lines available (Sunarto et al., 2011; Sano et al., 2004; Pikulkaew et al., 2009; Somga et al., 2010). It seems that a novel susceptible cell line to CyHV-3 can expand prior works. Moreover, early data described that the disease

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Abbreviations: KHV, Koi herpesvirus; **CyHV-3,** cyprinid herpesvirus-3; **IFA,** indirect immunofluorescence assay; **KoG,** koi gill cell; **CPE,** cytopathic effect.

of KHV are induced per gill infection *in vivo* (Miyazaki et al., 2008) and transient CPEs are caused in a common carp gill (CCG) cell line *in vitro* (Neukirch and Kunz, 2001). Their studies suggest that the gill cells may be a candidate for the development of cell line for KHV replication besides the commonly used fin-derived cell lines (Hedrick et al., 2000; Pikarsky et al., 2004; Davidovich et al., 2007; Dong et al., 2011; Sunarto et al., 2011).

According to the three complete genomes of KHV isolates from Japan (J), United states (U) and Israel (Aoki et al., 2007), KHV genotypes can be classified in accordance with the pattern of deletions and insertions of sequences among them (Kurita et al., 2009; Bigarre et al; 2009; Sunarto et al., 2011). Although, the epizootic of KHV has been reported in Taiwan (Tu et al., 2004), there have been no studies that compare differences in genome between Taiwanese and those three KHV strains as mentioned earlier (Kurita et al., 2009; Bigarre et al; 2009; Sunarto et al., 2011). Previous data indicated that in the enlarged thymidine kinase gene region, two Asian variants (A1 and A2) and seven European variants (E1-E7) were found in two lineages (Kurita et al., 2009). Besides, according to three domains in two regions, four genotypes of KHV isolates from different geographical distribution have been reported: the U/I genotype ($I^{-}II^{-}$), the J genotype ($I^{++}II^{+}$), the third genotype ($I^{-+}II^{+}$) from Poland and the Netherlands, and the fourth genotype ($I^{++}II^{-}$) from Indonesia (Bigarre et al; 2009; Sunarto et al., 2011). Recently, two major alleles in ORF146 gene have been found existing in the J-strain and the U/I strain (Dong et al., 2011). These results suggest that comparing the different gene regions of KHV can be used to realize the molecular lineage of the Taiwanese KHV isolate.

In this study, we report on the development of a gill cell line from koi and the characterization of the Taiwanese KHV isolate. Phylogenetic analysis of the enlarged thymidine kinase (TK) gene region, of alleles of the Asian lineage in the marker I and marker II regions, and of the ORF 136 gene indicates that the Taiwanese isolate was identical to a Japanese strain and related to the Asian genotype variant 1, with the $I^{++}II^{+}$ allele pattern and full-length 474-bp of the ORF136 gene.

MATERIALS AND METHODS

Primary culture and passages

A healthy one-year old healthy koi carp from a local farm was used for primary cell culture development. The fish was placed in flowing tap water for 2 min before being euthanized with MS-222. The gill arches were dissected, placed in 0.12% sodium hypochloride in PBS for 10 s, a 50 ppm iodine solution in PBS for 2 min, and 0.01% benzalkonium chloride in PBS for 2 min. The tissues were immersed in PBS containing gentamicin (1000 µg/ml) for 1 h at 4°C and washed twice with PBS. The tissues were digested with 0.25% trypsin-EDTA (Gibco, USA) 20 min at 25°C and the dispersed cells were harvested in a centrifuge tube on ice. The harvested cells were sieved through a stainless sieve and centrifuged at 300 *g* for 10 min at 4°C. The cell pellets were washed and centrifuged twice as aforementioned. The pellets were resuspended in Leibovitz L15 medium (L15) containing 10% fetal calf serum (FCS) and seeded in a 25 cm^2 flask. The new medium was changed every 7 days until the cells formed a confluent monolayer. The koi gill cells (KoG) were split at the ratio of 1:1 when reaching 80 to 90% area of the confluent monolayer. For storage, the cells were stored in a freezing medium consisting of 10% dimethyl sulphoxide (DMSO) and 90% FBS.

The cryotube (Nunc, Denmark) containing 2×10^6 cells was held at -70°C overnight and transferred to liquid nitrogen (-176°C). For viability test, the cryotube was thawed in a 25°C water bath and centrifuged to remove the freezing medium. After the cells were resuspended in L15 with 10% FCS, the viability test was determined using a trypan blue exclusion method. The rest cells were cultured in a 25-cm^2 flask.

Cell growth characterization

The RT-CES (real-Time Cell Electronic Sensing, Roche) was used for determining the optimum concentration of FCS and optimum medium used for the growth of KoG cells. Briefly, the KoG cells at subculture 40 were seeded at a density of 4×10^4 cells/well into a 16 E-plate which is integrated with microelectronic sensors at the bottom and incubated in 200 µL of culture medium at 25°C. For the effect of FCS concentration on the growth, four different concentrations of FCS (20, 10, 5 and 2%) in L15 were tested each on cells in four replicates. For the effect of medium on the growth, two types of medium (Eagle's MEM, L15) were tested each on cells with 10% FCS in four replicates. The cells were observed at 15-min intervals until 5 day. The doubling time was determined in the middle of the log phase of growth of cells cultured in L15 with 10% FCS per the RT-CES system's calculations.

Chromosome analysis

Chromosome analysis was done as described previously (Freshney, 2010). At passage 40, after 18 to 21 h of incubation, a 25-cm^2 flask of KoG cells of 80% confluence was treated with a medium containing cochicine (0.2 µg/ml, final) for 5 h at 25°C. The cells were then trypsinized, centrifuged and resuspended in 5 ml of 0.075 M KCl for 25 min at 25°C before being mixed with 5 ml of fresh, ice-cold Carnoy's fixative (methanol: acetic acid = 3:1) for 5 min and then centrifuged at 200 *g* for 2 min at 4°C. The supernatant was discarded, and the cells were resuspended in 5 ml of ice-cold Carnoy's fixative and left on ice for 10 min, then centrifuged again and resuspended in 0.2 ml of fresh Carnoy's fixative. The cell suspension was drawn, dropped onto cold slides, and dried rapidly over a beaker of boiling water. The slide was stained with a 10% Giemsa stain and examined under a microscope.

Production of antiserum against KHV envelope protein

A truncated recombinant protein was expressed and used as an antigen to produce a rabbit-anti KHV serum (unpublished data). Briefly, the primers were designed to amplify truncated ORF81 gene fragment coding 88 amino acids from C-terminal of KHV ORF81 protein according to the Genbank data on AP008984 for KHV. The full-length KHV ORF81 gene was amplified by PCR using the genomic DNA from a KHV-infected fish as a template, with the 5'-forward primer added as a *Bam*HI restriction sequence (underlined) (5'-ATGGATCCATGGCAGTCACCAAAG-3') and the 3'-reverse primer added as a *Xho*I restriction sequence (underlined) (5'-ATCTCGAGCCACATCTTGCCGGTG-3'). PCR cocktails were mixed using a Takara LA-Taq polymerase kit (Takara, Japan) and reaction conditions were done as follows: one cycle at 95°C for 5 min; 95°C for 30 s, 58°C for 45 s, 72°C for 1 min, 30 cycles; and a final extension at 72°C for 7 min. The PCR products were purified

using a PCR purification kit (QIAGEN, USA), digested with two restriction enzymes and then inserted into the B+X digested pET29a transfer vector (Novagen, USA), generating pET29a-KHV-ORF81. The recombinant DNA was transformed into Novablue *E. coli* competent cells, which were then used to propagate the plasmid constructs. The transformants were selected on Luria-Bertani (LB) plates containing 30 µg/ml kanamycin. The recombinant plasmid DNA was prepared using a plasmid DNA QIAprep Spin Mini Kit (QIAGEN, USA) and the presence of the OEF81 fragment was confirmed by DNA sequencing. Subsequently, the fragment was further excised from the pET29a vector with *Sac*I and *Xho*I, and then inserted to an S+X digested pET29a.

A construct containing truncated ORF81 gene (264-bp) was transformed into BL21 (DE3) host cells. A single colony selected from the LB plate containing 30 µg/ml of kanamycin was first grown in 2 ml of LB broth (30 µg/ml of kanamycin) with shaking at 225 rpm at 37°C overnight. After adding 1 ml of the overnight-incubated broth to 100 ml of LB broth, the single colony was grown to an optical density of 0.4 to 0.6. Protein expression was induced by adding isopropyl- β-D-thiogalacto-pyranoside (IPTG) to the 100 ml of LB broth (of a final concentration of 0.5 mM) and collected 4 h after being induced at 37°C. The expressed recombinant protein was purified using His-bind columns and a Ni-NTA buffer kit as per the manufacturer's instructions (Merck, USA). The polyclonal antibody was raised against the recombinant protein using rabbits as described (Johnstone and Thorpe, 1988).

Susceptibility of the KoG cell line to koi herpesvirus and indirect immunofluorescence assay

At passage 40, the cells were tested for susceptibility to the KHV strain TW BL98 isolated from laboratory-developed koi fin (TKF) cells (unpublished data). A flask of 25 cm^2 of KoG cells was inoculated with KHV at 2 to 4 × 10^5 $TCID_{50}$/ml and observed daily for cytopathic effect (CPE) under a light microscope. In addition, an indirect immunofluorescence assay (IFA) was used to further confirm the infection of KHV in KoG cells. Briefly, 2 to 4 × 10^5 cells/ml were seeded to glass coverslips in each well of a 12-well plate. After 18 to 24 h, the cells were inoculated with KHV at 2 to 4 × 10^4 $TCID_{50}$/ml. The glass coverslips were removed at 1, 4, 5, 8 days post infection, fixed in iced 80% acetone in distilled water for 10 min, air-dried, incubated with an rabbit antiserum against a truncated recombinant protein diluted 1:100 for 1 h at 37°C and washed with PBS twice. The coverslips were incubated with FITC-labeled goat anti-rabbit IgG (H+L) (KPL, USA) diluted 1:1,000 for 1 h at 37°C and washed with PBS twice. They were then covered with a T-pro mounting reagent containing 0.1% 4'-6-Diamidino-2- phenylindole (DAPI) (SB, Taiwan) and observed with a fluorescent microscope.

Electron microscopy

The supernatants of KoG cells 4 days post infection (dpi) were centrifuged at 16,000 *g* for 10 min by Airfuge (Beckman) and re-suspended pellets were stained with equal volume of 2% phosphotungstic acid for 2 min. The stained virions were absorbed onto the grids (Agar, UK), and observed using a transmission electron microscope (JEOL JEM-1400, Japan) at 100 Kv. The same cells were fixed with 2.5% (v/v) glutaraldehyde in a 0.1 M phosphate buffer and post-fixed in 2.0% (w/v) osmium tetraoxide, embedded in resin, ultra-thin sectioned, stained with uranyl acetate and lead citrate, and examined using the aforementioned apparatus.

KHV replication in KoG cells

Briefly, 2 to 4 × 10^5 cells/ml were seeded to each well of a 24-well plate with an L15 containing 10% fetal calf serum, 100 IU penicillin /ml and 100 µg streptomycin/ml. After 18 to 24 h, the confluent cells were inoculated with KHV at 2 to 4 × 10^4 $TCID_{50}$/ml, incubated at 25°C for 2 h and washed twice with an L15. The inoculated cells were cultured with L-15 medium containing 2% fetal calf serum with antibiotics. The supernatants of duplicate wells were removed from day 1 until day 11 post infection, and stored at -80°C for extracellular virus titration. The adherent cells in duplicate wells were digested with 0.25% trypsin-EDTA, removed, suspended in 1-ml of L15 containing 2% FCS, frozen and thawed 3 times, and stored at -80°C for cell-associated virus titration. After day 11, the titers of extra- and cell-associated viruses were analyzed using the $TCID_{50}$ method (Reed and Muench, 1938).

Phylogenetic analysis

Based on previous studies (Kurita et al., 2009; Bigarre et al; 2009; Dong et al., 2011), four variable regions of the KHV genome were chosen to analyze the relationship between the Taiwanese strain (T-strain) and other complete genomes of CyHV-3. Both the larger region of the TK gene and the ORF136 gene were used to differentiate the European lineage from the Asian lineage as described in previous reports (Kurita et al., 2009; Dong et al., 2011). The duplex PCR targeting marker I (the region between the ORF29 and ORF30 genes) and marker II (the region near to the start of ORF133) was used to distinguish Asian KHV from other area isolates (Bigarre et al., 2009). The primers for those four regions are shown in Table 1. The 50 µL of PCR reaction mixture included 5µL of 10x PCR buffer, 8 µL of 2.5 mM dNTP mixture, 0.5 µL (2.5 U) of LA-Taq DNA polymerase (TaKaRa, Japan), 1 µL of 10 µM of each primer, 2.5 µL of template DNA and 12 µL of sterilized distilled water. The PCR conditions for the previous three regions were as follows: one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 45 s, 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR condition for the 136 gene was the same as described previously except that the annealing temperature was lowered to 52°C. The PCR products were ligateded into the vector using TA cloning kit (Yeastern Biotech, Taiwan) and transformed into the *Escherichiae coli* strain DH5. At least three independently derived clones were sequenced by an ABI PRISM 377 DNA sequence (Applied Biosystems, USA).

The aforementioned sequences of amplified genes were aligned to the three complete genome of KHV [KHV-J(AP008984), KHV-U(DQ657948) and KHV-U(DQ1773346)] in GenBank using the BLAST algorithm provided by the National Centre for Biotechnology Information (NCBI).

RESULTS

Establishment of KoG cell line

In this study, a koG cell line was obtained using gills from adult koi. The primary cells formed a confluent monolayer after 6 weeks. Chromosome analysis shows that the number of KoG cell lines at passage 46 ranges from 30 to 53 with a modal distribution of 43 (Figure 1). Morphologically, monolayer cells consisted of epithelial-like and fibroblastic-like cells (Figure 2). At passage 1, the elongated fibroblastic-like cells grew more abundantly than those in passage 20 (Figure 2). The cells grew slowly and were subcultured at a 1:1 ratio at 7 to 10 day intervals in an L15 supplemented with 10% FCS. After passage 30, the KoG cells were subcultured at 5 to 6 day intervals. The cells at subculture 30 were frozen in liquid nitrogen for two months and used for viability test. The survival

Table 1. Nucleotide sequences of primers for genotyping in this study.

Primer	Nucleotide sequence (5'-3')	References
KHV-tkF (sense)	AACGCGGGCCAGCTGAACAT	Kurita(2009)
KHV-tkR (antisense)	TGTGTGTATCCCAATAAACG	Kurita(2009)
KHV-markerIF (sense)	CTACTCAGGAGCCATCATCATCG	Bigarre (2009)
KHV-markerIR (antisense)	AGGACTTGGTAGGTGCCTCC	Bigarre (2009)
KHV-markerIIF (sense)	GCTCATTTTAGCGCTTCTGTG	Bigarre (2009)
KHV-markerIIR (antisense)	CGCTGCCTACCCAATTCGCT	Bigarre (2009)
KHV-orf136F (sense)	AATGAAGGCCTCTAAACTGC	Dong (2011)
KHV-orf136R (antisense)	TTAGATTTTTCTAAAGTGCACG	Dong (2011)

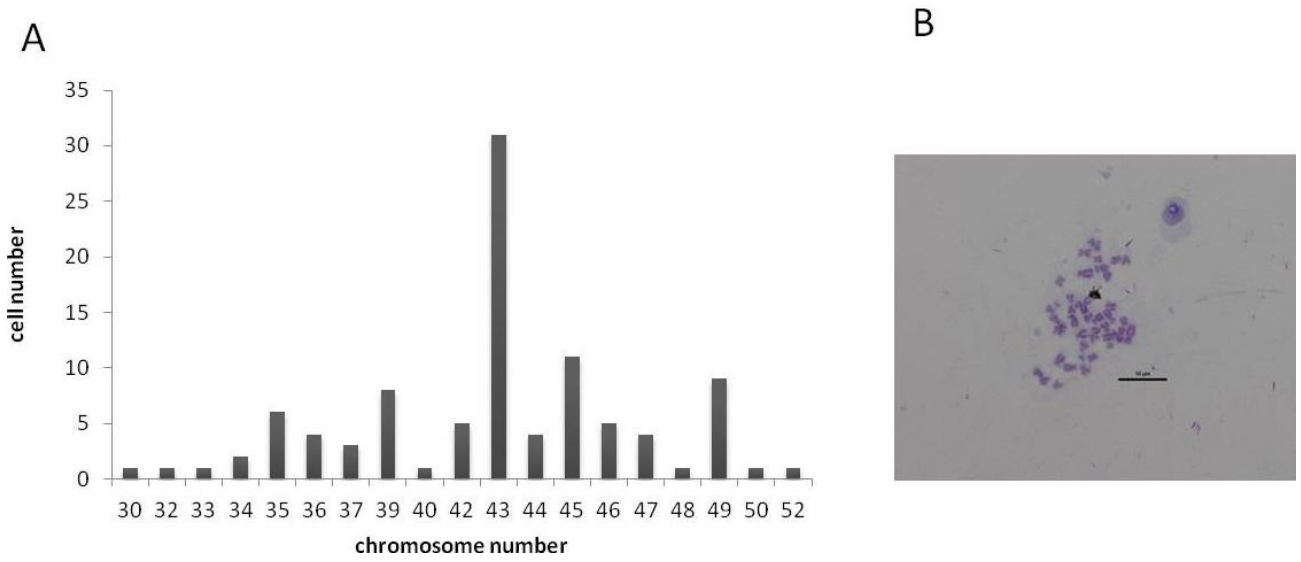

Figure 1. Distribution of chromosomes number (A) and metaphase of KoG cells (B) at subculture 46 (n = 100 cells).

rate of KoG cells was 70 to 75% in L-15 with 10% FCS at 25°C. The KoG cell line was subcultured for more than 56 passages.

Cell growth characterization

Figure 3A demonstrates that the growth rates of KoG cells in L15 containing 10 or 20% are much higher than those of KoG cells in the same medium with 2 or 5% FCS. As can be seen in Figure 3B, the L15 gave the satisfactory result in the growth curve of KoG cells comparing with MEM. The doubling time was calculated as 43.8 ± 0.8 h according to the middle of log phase ranging from 48 to 56 h in the growth curve of the KoG cells cultured in L15 containing 10% FCS as shown in Figure 3A.

Susceptibility of KoG cells to KHV

The cytopathic effects (CPEs) were illustrated sequentially after KoG cells were infected with CyHV-T98. After infection, CPEs appeared as few vacuoles in cytoplasm at 1 day post-infection (dpi) (Figure 2D1). From 2 dpi, the cells showed syncytia formation and extensive vacuolization. Some of the vacuoles fused together to form a large one (Figure 2D2). Refractive rounding cells were found at 3 dpi (Figure 2D3), and cell sloughing with lysis appeared at 4 dpi (Figure 2D4). At 6 dpi, the confluent monolayer was severely detached and lost its structure (Figure 2D6). The results are similar to those of earlier studies (Pikasky et al., 2004; Dong et al., 2011; Sunarto et al., 2011). The CPEs caused by KHV in KoG cells were further confirmed using an IFA as shown in Figure 3. At 1 dpi, a few cells with vacuoles in cytoplasm were detected using a rabbit-anti KHV serum (Figure 4A). At 4 dpi, more than 75% of cells were detached from their glass cover slips, and the residual attached cells were detected with large vacuoles in cytoplasm and syncytia formation (Figure 4B). At 6 dpi, the control cells showed slightly fluorescent in cytoplasm with intact nuclei (Figure 4C). It is evident that these findings are in good agreement with those in Figure 2. The electron microscopy examination of KoG cells infected with KHV can be seen in Figure 5. The mature virus had an icosahedral shape with an

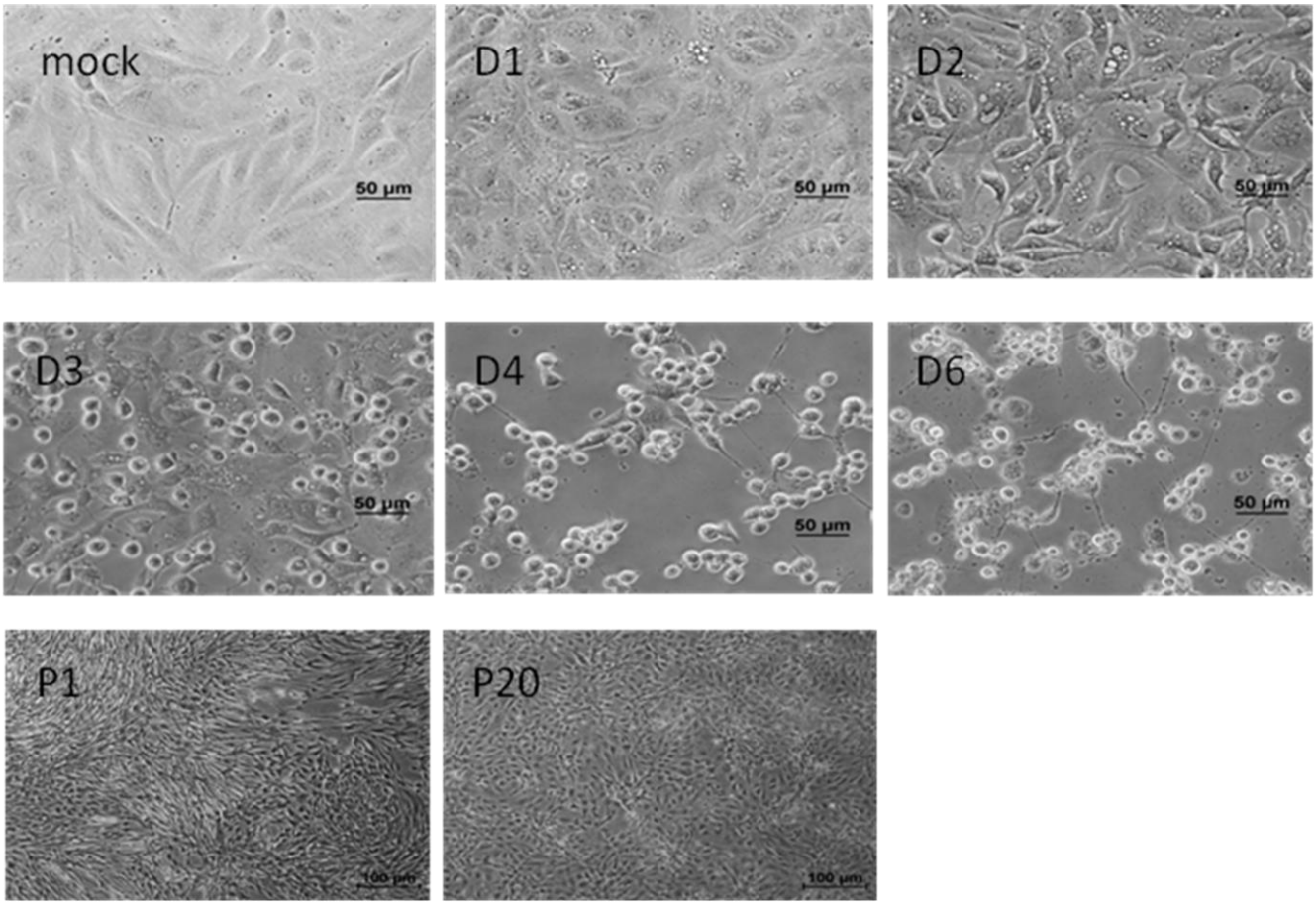

Figure 2. The CPE of KoG cells at passage 40 observed at 1, 2, 3, 4 and 6 days after infection of KHV. The morphology of un-infected passage 1 (P1) and passage 20 (P20) KoG cells are shown.

envelope in the negative staining (Figure 5A).

In the thin section, mature and immature virons were observed in the cytoplasm (Figure 5B). These results are consistent with those in the CPEs (Figure 2) and the IFA (Figure 4).

Replication of KHV in KoG cells

As shown in Figure 6, the titers of CyHV-T98 in the inoculated KoG cells were measured extra- and endocellularly using $TCID_{50}$ methods. The titer of extracellular viruses in the supernatant reached its highest value of $10^{4.5}$ $TCID_{50}$ ml^{-1} at 5 dpi and the titer of cell-associated viruses ranged from $10^{1.4}$ to $10^{2.2}$ virus $TCID_{50}$ ml^{-1} during the 11 dpi. The highest total virus titer was $10^{6.1}$ $TCID_{50}$ ml^{-1} at 5 dpi. At 6 dpi, the titer of extracellular virus in the supernatant dropped from $10^{4.5}$ to $10^{3.6}$ $TCID_{50}$ ml^{-1} and never increased throughout the experiment. Comparing Figures 2 and 4 shows that 5 days of incubation should be suitable for the replication of KHV in KoG cells at 25°C.

Phylogenetic analysis

In this study, the sequence of the CyHV-T98 strain (T-strain) was compared with three complete genomes of CyHV-3 deposited at the GenBank as follows: J (Japanese) strain (AP008984), U (United States) strain (DQ657948) and I (Israel) strain (DQ177349). In the enlarged TK gene region, two genotypes, Asian (variant A1-A2) and European (variant E1-E7) were found due to the genetic variations worldwide (Kurita et al., 2009).

Table 2A illustrates that an AT deletion of 10 nucleotides upstream of the termination codon was identified in the T- and J-strains, which could be translated into eight more amino acids (24 nt) by the frame-shift existing in all Asian genotypes compared to the European genotype (U-strain and I-strain). As shown in Table 2A, seven “T” bases in the serial “T” region (No. 877 to 885) indicated that the T-strain was similar to the J-strain classified as Asian genotype variant 1. In addition, only single nucleotide polymorphism was observed in the serial “A” region (No. 949 to 956), in which a single base was changed at position 949 from A to T. It is apparent that KHV from

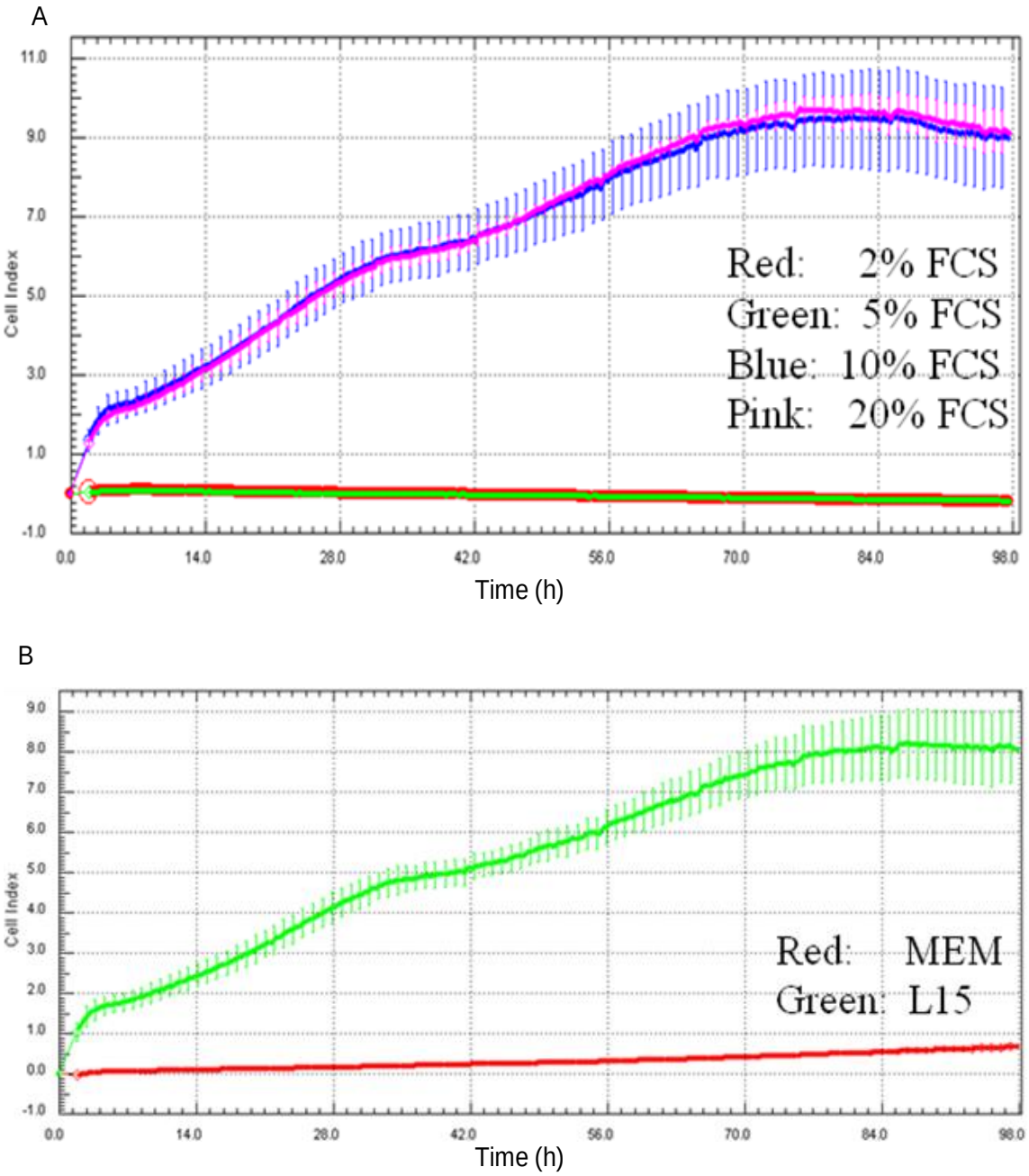

Figure 3. Real-time monitoring of (A) optimum concentrations of FCS and (B) optimum media treatment on KoG cells. Each color represents a different concentration or medium in the graphs.

Taiwan is closely linked to the Asian KHV linage. Based on three variable domains in two regions of KHV, three genotypes, including U/I, J, and an intermediate between U/I and J were classified using a duplex PCR assay (Bigarre et al., 2009). As shown in Table 2B, the marker I region indicated that an allele I^{++} with the full-length 168-bp sequence found in the T-strain and the J-strain compared to an allele $I^{-}$with 38-bp deletion of U/I strain.

In Table 2C, the marker II region displayed two alleles: allele II^{+} (352-bp sequence) in T- and J-strains, and allele II^{-} with a 74-bp deletion (U/I-strains). The results showed that the Taiwanese isolate demonstrated the I^{++} II^{-} genotype, which is identical to the J-strain (I^{++} II^{-}) of the Asian lineage.

In the ORF136 gene, two major alleles have been observed: the full- length 474-bp sequence (J-strain) and the 12-bp deletion sequence (U/I-strain) (Dong et al., 2011). The full-length 474-bp sequence of the T-strain was identical to that of the J-strain except a nucleotide variation was found at nt 30 from T to C. Compared to the

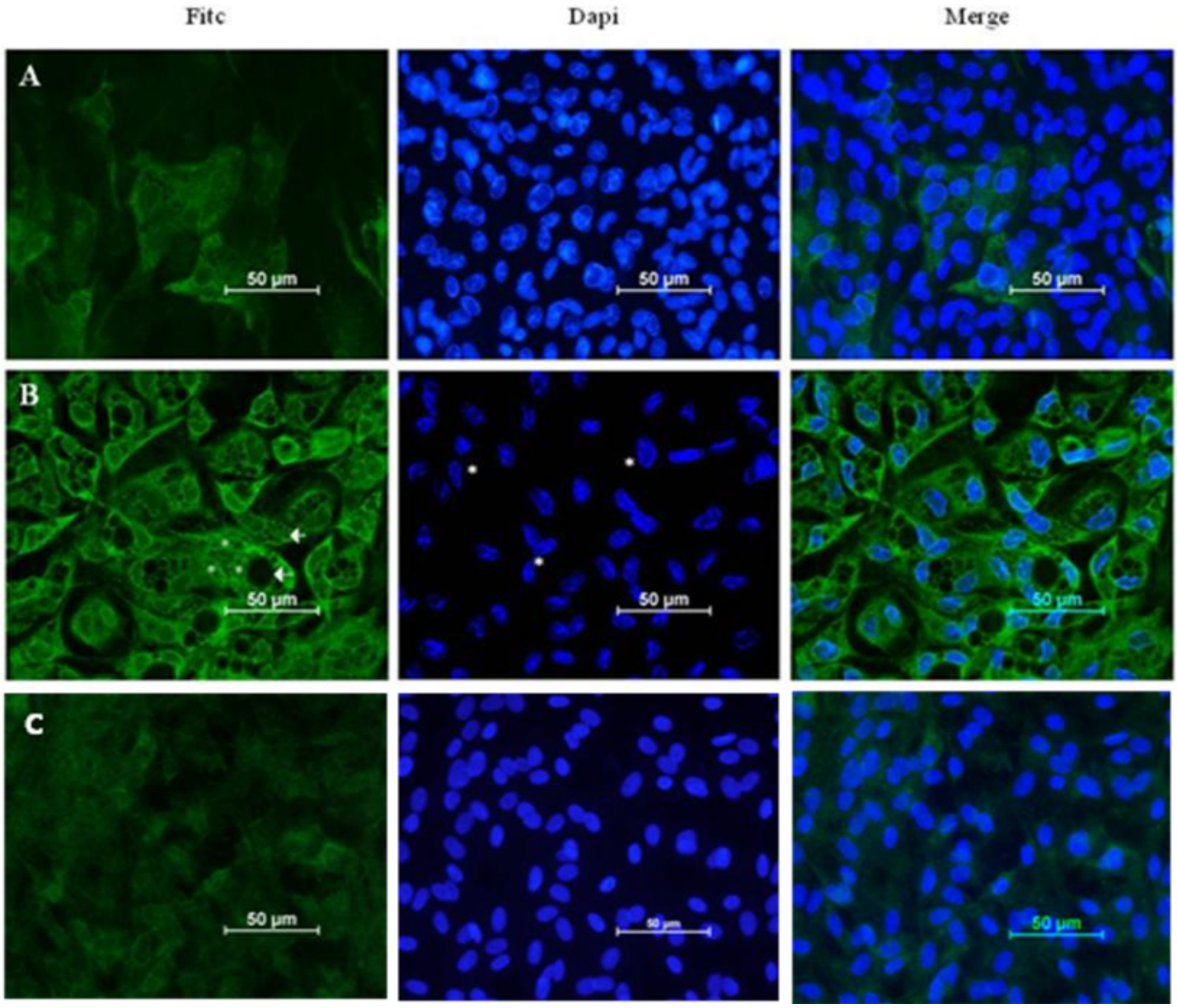

Figure 4. Imunofluorescence photographs of KoG cells infected with KHV under fluorescent light microscope. The KHV-infected cells were stained at 1 dpi (A) and 4 dpi (B) using rabbit anti-KHV ORF81 serum and FITC-congugated goat anti-rabbit IgG antiserum. The nucleoli were stained with DAPI. At 4 dpi (B), the syncytial cells (asterisk) showed different-sized vacuoles (arrow) in the left panel and deformed nuclei with the margination of chromatin (asterisk) in the middle panel. The control cells were stained at 6 dpi (C) with normal cell shape and nucleoli.

T- and J-strains, the U/I-strain contained a 12-bp deletion allele. This indicates that the Taiwanese isolate was related to the typical Asian genotype.

DISCUSSION

Data from previous studies indicate that cell lines derived from carp tissues, including the brain, gills and fins, result in the isolation of KHV. These cells are originated from freshwater cyprinid fish (common carp, koi, silver carp and gold fish), including fin-derived KF-1, KFC, Tol/FL, Au, KCF-1,KT-2 (Hedrick et al., 2000; Perelberg et al., 2003; Pikarsky et al., 2004; Davidovich et al., 2007; Dong et al., 2011; Sunarto et al., 2011), brain-derived CCB (Neukirch and Kunz, 2001) and gill-derived CCG cell lines (Neukirch and Kunz, 2001). However, the transient CPEs were observed in KHV-infected common carp gill (CCG) cell line 10 to 11 days post infection but vanished two weeks post infection (Neukirch and Kunz, 2001). In this study, we have developed a cell line derived from the koi gills suitable for KHV replication. During our development of cell lines, several gill cell lines were developed

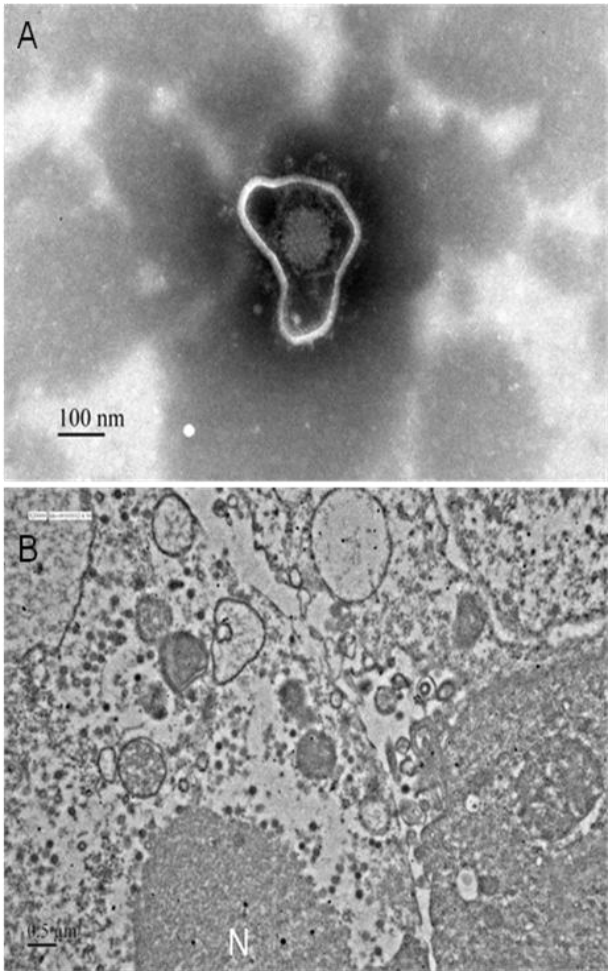

Figure 5. Electron micrograph of the KHV in KoG cells. (A): negative staining of mature enveloped virion from the supernatant of KHV-infected KoG cells; (B): mature and immature virons in the cytoplasm of KHV-infected KoG cells. N: nucleus.

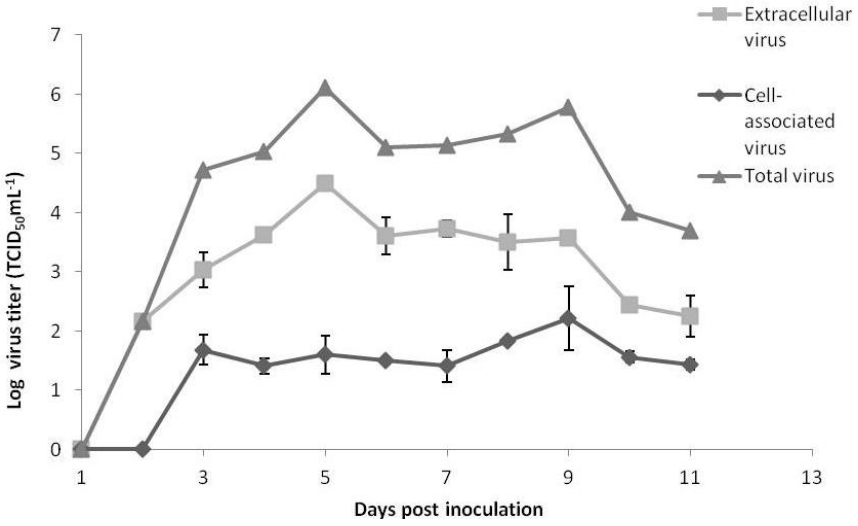

Figure 6. Multistep growth curve of KHV in KoG cells. The data indicate the mean ± standard deviation of two independent assays.

Table 2. Sequence alignment of the Taiwanese KHV isolate with three type strains from Japan, USA and Isreal (KHV-J, KHV-U and KHV-I). Compared with the enlarged TK gene region, Taiwanese strain was classified as A1 variant. Aligned with marker 1 and marker 2, Taiwanese displayed the $I^{++}II^{+}$ allele genotype. The Taiwanese isolate was also related to he Asian lineage according to the alignment of ORF136. The motifs are underlined. Stop codons are denoted as rectangles.

A. Alignment of enlarged TK gene

T-strain TW BL98	796	CCTCGTTCTTGTAAC----ATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAATAATGTGCGA
J-strain (AP008984)	796	CCTCGTTCTTGTAAC----ATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAATAATGTGCGA
I-strain (DO177346)	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAAT----TGTGCGA
U-strain (DQ657948)	796	CCTCGTTCTTGTAACATATCTATCCTGTGATGGTGTGTGTGGAACCAATAAAAT----TGTGCGA

Alignment of enlarged TK gene

T-strain TW BL98	839	CAATAAAATAATGTGCGACTTGAATATGGTTGTACGGGTTTTTTT----AACAAAAACTAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTTTGCGTCCAATACTTT----TAAAAAAA-----GGAGATATTAAATATAGT
J-strain (AP008984)	839	CAATAAAATAATGTGCGACTTGAATATGGTTGTACGGGTTTTTTT----AACAAAAACTAAACTACCGAAACACGAAACACTTGCTCGGAGCGACTTTGCGTCCAATACTTT----AAAAAAAA----GGAGATATTAAATATAGT
I-strain (DO177346)	841	CAATAAAAT----TGTGCGACTTGAATATGGTTGTACGGGTTTTTTT----AACAAAAACTAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTTTGCGTCCAATACTTT-TTAAAAAACA----GGAGATATTAAATATAGT
U-strain (DQ657948)	841	CAATAAAAT----TGTGCGACTTGAATATGGTTGTACGGGTTTTTTTTTAACAAAAACTAAACTACCGAAACACGAAACACTTGCTCTGAGCGACTTTGCGTCCAATACTTT-----AAAAAAAACAGGAGATATTAAATATAGT

Table 2B. Alignment of marker I.

T-strain TWBL98	1	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAGACAACCACCTCG
J-strain (AP008984)	1	CTACTCAGGAGCCATCATCGGCAACCTCAACCCCGGCAGCCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCGGAGTCGTCCACGATGGTGACACCCGAGACAACCACCTCG
I-strain (DO177346)	1	CTACTCAGGAGCCATCATCGGCAA----------CCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCG----------CGAGACAACCACCTCG
U-strain (DQ657948)	1	CTACTCAGGAGCCATCATCGGCAA----------CCTCAACCCCGGCAGCCTCAACTTCACCTTCAGAATCTTCAACGGTATGGATGATAGAGTCG----------CGAGACAACCACCTCG

Table 2C. Alignment of marker 2.

T-strain TWBL98	51	TAAATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGAGGTCGATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCT
J-strain (AP008984)	51	TAAATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCTCGAGGTCGATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCT
I-strain (DO177346)	51	CAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCT
U-strain (DQ657948)	51	CAA----------ATGAACAGAAAGAAAGCTCAGAAGTGAGATGGGCCAGAAGCACGACAAGTACCAGGGCGCCGATCT

Table 2D. Alignment of ORF136 gene

T-strain TW BL98	28	TATATGGGTGGCTTTCGCGGCCGGCCAGAACGCCACCACCCTGGCTCCGCTCGCCGCCACTAACGGCACAACAACCATGAACTCTACCTGGTCCTCTACCGCTT
J-strain (AP008984)	28	TACATGGGTGGCTTTCGCGGCCGGCCAGAACGCCACCACCCTGGCTCCGCTCGCCGCCACTAACGGCACAACAACCATGAACTCTACCTGGTCCTCTACCGCTT
I-strain (DO177346)	28	TACATGGGTGGCTTTCGCGGCCGGCCAGAACGCCACCACCCTGGCTCCGCTCGCCGCCACTAACGGCACAACAACCATGAACTCTACC----------GCTT
U-strain (DQ657948)	28	TACATGGGTGGCTTTCGCGGCCGGCCAGAACGCCACCACCCTGGCTCCGCTCGCCGCCACTAACGGCACAACAACCATGAACTCTACC----------GCTT

but none of them was susceptible to KHV. In consideration of the epitheliotropism of alloherpesviruses (Smail and Munro, 2012), we decided to change the FCS concentration from 20 to 10% in the beginning medium for the primary culture, expecting to inhibit fibroblast cells growth in our cell line. This modification is based on the fact that the major serum growth factors of the FCS are from platelets, which can stimulate the fibroblasts growth and inhibit the epithelial cells growth (Freshney, 2010). According to results from Figure 3, we have established a koi gill cell line (KoG) cultured in an L15 supplemented with 10% fetal calf serum. The cells consisted of fibroblast-like and epithelium-like cells. Although, the cells grew slowly with a split ratio of 1:1 at first, their susceptibility to CyHV-3 was displayed by typical CPEs, including vacuoles in cytoplasm and syncitia formation as described previously (Pikarsky et al., 2004; Sunarto et al., 2011).

In addition, compared with CCB cells, the infected KoG cells with larger vacuoles in CPE could be easily counted and applied to the plaque assay (data not shown). Further, the methods, including an IFA and electron microscopy, provided more results that were comparable to those of earlier studies (Pikarsky et al., 2004; Dong et al., 2011; Sunarto et al., 2011). It is evident that a KoG cell line is established and useful for the proliferation of CyHV-3. Earlier works demonstrate that the sequence analysis of four variable regions of KHV genome can discriminate between genotypes of KHV isolated from different countries (Kurita et al., 2009; Bigarre et al; 2009; Dong et al., 2011). In this study, comparing our strain with the J-strain and the U/I-strain showed that our strain was related to the Asian genotype variant A1 identical to the J-strain in the larger TK gene region, with only one nucleotide polymorphism. This is similar to previous reports that two genotypes of KHV (A1 and A2) exist in Taiwan (Kurita et al., 2009). Regarding the variation of two molecular markers, four alleles have been reported with their corresponding geographical distribution: I^{-} $^{-}II^{-}$ (United States and Israel), I^{-} $^{+}II^{-}$ (Netherlands and Poland), $I^{++}II^{-}$ (Indonesia) and $I^{++}II^{+}$ (Japan) (Bigarre et al., 2009; Sunato et al., 2011). As shown in our results, the allele $I^{++}II^{+}$ of our isolate is consistent with that of the J-strain (Table 2B and C). A recent report on the ORF 136 gene provided the third differentiation between two CyHV-3 lineages (U/I and J) (Dong et al., 2011). The full-length 474-bp sequence of T-strain was identical to that of the J-strain (Asian lineage) except for a nucleotide variation.

Based on our results, this demonstrates that the Taiwanese KHV isolate has an $I^{++}II^{+}$ allele genotype of the Asian lineage and is closely related to the J-strain (Asian genotype variant 1).

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