Vol. 105: 1–8, 2013
doi: 10.3354/dao02594

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 9

Megalocytivirus infection in orbiculate batfish *Platax orbicularis*

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ABSTRACT: Megalocytiviruses cause systemic disease in both marine and freshwater fishes, negatively impacting ornamental and food fish aquaculture. In this report, we characterize a megalocytivirus infection in a captive marine ornamental fish, the orbiculate batfish *Platax orbicularis*. Histologic examination revealed cytomegalic cells characterized by strongly basophilic granular intracytoplasmic inclusions within various organs. Transmission electron microscopy revealed icosahedral virus particles within the cytoplasm of cytomegalic cells consistent with an iridovirus infection. Analysis of the major capsid protein gene sequence confirmed that the orbiculate batfish virus is a member of the family *Iridoviridae* and is identical to the only other megalocytivirus reported from a marine ornamental fish, the Banggai cardinalfish *Pterapogon kauderni* iridovirus.

KEY WORDS: Iridovirus · Megalocytivirus · Orbiculate batfish · Phylogeny

INTRODUCTION

Iridoviruses have been identified from various species of fishes and are important problems for aquaculture industries (Ahne et al. 1997, Rodger et al. 1997, He et al. 2000, Paperna et al. 2001, Wang et al. 2003, Shi et al. 2004, Wang et al. 2011). Members of the family *Iridoviridae* infect a range of poikilothermic hosts, including invertebrates, fish, amphibians, and reptiles (Chinchar et al. 2009). The family is organized into 5 genera, *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus*; the latter 3 genera infect fishes. Megalocytiviruses are large, double-stranded DNA viruses with an icosahedral capsid (Kurita & Nakajima 2012). Members of the genus *Megalocytivirus* are pathogenic to a wide variety of freshwater and marine fishes, and have had a negative impact on ornamental and food fish aquaculture worldwide (Armstrong & Ferguson 1989, Anderson et al. 1993, Fraser et al. 1993, Rodger et al. 1997, Sudthongkong et al. 2002, Gibson-Kueh et al. 2004, Jeong et al. 2008a,b, Chinchar et al. 2009, Weber et al. 2009, Kim et al. 2010, Yanong & Waltzek 2010, Zhang et al. 2011). Megalocytiviruses induce lethal systemic disease at water temperatures ranging from 7.5 to 32°C (45.5 to 89.6°F) (Chen et al. 2003,

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Yanong & Waltzek 2010, Wang et al. 2011, Zhang et al. 2011, Waltzek et al. 2012). Epizootics may result in up to 100% mortality under intensive aquaculture conditions (Fraser et al. 1993, Rodger et al. 1997, He et al. 2000, Sudthongkong et al. 2002). These viruses can spread horizontally from fish to fish by cohabitation, exposure to water or equipment carrying the virus, and ingestion of infected fish or feed items (He et al. 2002, Go & Whittington 2006, Yanong & Waltzek 2010).

Megalocytivirus-infected fish may exhibit nonspecific clinical signs including lethargy, anorexia, hyperpigmentation, exophthalmos, skin lesions, unusual swimming behavior, severe anemia, and white feces (Chen et al. 2003, Weber et al. 2009, Wang et al. 2011, Waltzek et al. 2012). On post-mortem examination, fish infected with megalocytiviruses may exhibit hemorrhagic lesions, renomegaly, splenomegaly, hepatomegaly, and coelomic distension resulting from hemorrhagic fluid accumulation (Chen et al. 2003, Weber et al. 2009, Yanong & Waltzek 2010, Wang et al. 2011, Zhang et al. 2011, Waltzek et al. 2012). Histopathologic examination typically reveals cytomegalic mesenchymal cells characterized by strongly basophilic or amphophilic granular intracytoplasmic inclusions observed in multiple organs including the spleen, kidney, liver, heart, brain, gills, intestine, eyes, and gonads (Chen et al. 2003, Gibson-Kueh et al. 2003, Weber et al. 2009, Yanong & Waltzek 2010, Zhang et al. 2011, Waltzek et al. 2012). Transmission electron microscopy (TEM) invariably reveals numerous icosahedral virus particles with a capsid diameter between 120 and 200 nm in the cytoplasm of infected cells (Chen et al. 2003, Weber et al. 2009, Yanong & Waltzek 2010, Wang et al. 2011, Zhang et al. 2011, Kurita & Nakajima 2012, Waltzek et al. 2012).

Phylogenetic analyses support 4 separate species within the genus *Megalocytivirus* (Kurita & Nakajima 2012, Waltzek et al. 2012). Megalocytiviruses related to red sea bream iridovirus (RSIV) cluster into 1 of 2 genotypes and have been associated with mass mortality epizootics in more than 30 maricultured species in Japan, Korea, China, and Southeast Asia (Inouye et al. 1992, Jung & Oh 2000, Kawakami & Nakajima 2002, Wang et al. 2003). Infectious spleen and kidney necrosis virus (ISKNV), originally isolated from mandarin fish *Siniperca chuatsi* raised for food in China, has also resulted in epizootics in more than 10 species of freshwater ornamental fishes (He et al. 2000, Paperna et al. 2001, Sudthongkong et al. 2002, Yanong & Waltzek 2010). A second ISKNV genotype was recently recognized following disease episodes in ornamental and food fish species (Kurita & Nakajima 2012), which included Banggai cardinalfish iridovirus (BCIV) isolated from a marine ornamental species (Banggai cardinalfish *Pterapogon kauderni*) and marbled sleepy goby iridovirus (MSGIV) isolated from a freshwater species (marbled sleeper goby *Oxyeleotris marmorata*) cultured for food in China (Weber et al. 2009, Wang et al. 2011). The third species, turbot reddish body iridovirus (TRBIV), has primarily been associated with disease in a cultured Asian flatfish species, the turbot *Scophthalmus maximus* (Shi et al. 2004). A fourth megalocytivirus species was recently proposed, threespine stickleback iridovirus (TSIV), from an epizootic that occurred in a Canadian collection of threespine stickleback *Gasterosteus aculeatus* (Waltzek et al. 2012).

Orbiculate batfish *Platax orbicularis* are tropical marine ornamental species belonging to the order Perciformes, family Ephippidae. They can be found throughout the Western Indo-Pacific region (Capuli & Ortanez 2011). Orbiculate batfish occur in a variety of habitats: mangroves in shallow coastal waters, other sheltered waters, deep seaweed reefs, and open waters. They prefer water temperatures ranging from 22 to 28°C (71.7 to 82.4°F) (Capuli & Ortanez 2011). Orbiculate batfish have only recently been successfully bred in captivity, and thus information regarding viral diseases in this species is limited (David et al. 2010). Only 2 viral infections, lymphocystis and betanodavirus, have previously been reported in *P. orbicularis* (Lawler et al. 1978, David et al. 2010).

In the present study, we report a megalocytivirus infection in *Platax orbicularis* that was associated with mortality in recently imported wild-caught fish acquired by a public aquarium in Belgium. The objective of this study was to compare clinical aspects of the disease as well as microscopic and ultrastructural features of the virus with previously described megalocytiviruses, including the virus associated with the only other marine ornamental epizootic reported in Banggai cardinalfish.

MATERIALS AND METHODS

Clinical history

A mortality event occurred in March 2010 among a group of 10 orbiculate batfish quarantined at a public aquarium in Belgium. These wild-caught juvenile

orbiculate batfish (average of 12 cm in total length) from Indonesia had been purchased by an ornamental fish import facility in the UK and sent to a quarantine facility in the UK, where they were acclimatized and quarantined prior to delivery to the aquarium in Belgium in February 2010. At the aquarium, the batfish were maintained in a quarantine system equipped with an external power filter. Water quality parameters were maintained at a temperature of 24°C (75°F), pH 7.8, nitrite <0.3 mg l^{-1}, nitrate 0 to 12.5 mg l^{-1}, salinity 32, 96% oxygen saturation, and undetectable total ammonia nitrogen. The orbiculate batfish were fed mysid (*Mysidopsis*) and brine shrimp (*Artemia*) 5 to 6 times per day.

On arrival, several fish had moderately frayed fins. Two days after arrival, one fish became anorexic, displayed an increased respiration rate, and was noticeably darker than other batfish in the same tank. A wet mount examination of skin mucus of this batfish was negative for parasites. This fish was dipped in freshwater for 3 min d^{-1} for 2 d to help remove any parasites not detected by wet mount examination. All fish in the same tank were treated with a formalin bath at 100 mg l^{-1} for 6 h to control a suspected parasite infection. Fish were also treated prophylactically with an oxytetracycline (Aquatet®, PHARMAQ) bath at 100 mg l^{-1} daily for 7 d. Mortality began during the treatment and a total of 8 fish died during the 4 wk quarantine period. Gross necropsy findings were unremarkable; however, fleshy white nodules were noted on the dorsal fins of all fish that died and were also observed on the remaining 2 fish in the tank. Following necropsy, specimens were fixed in 10% neutral buffered formalin and sent to the International Zoo Veterinary Group (IZVG) Pathology Laboratory, UK, for histological processing and examination.

Histopathology

For 2 separate formalin-fixed fish that had been necropsied, 5 transverse sections and 1 sagittal section of the head were cut and dehydrated through a series of water and alcohol mixtures to full alcohol. Tissues were then cleared through a clearant and embedded into paraffin blocks (2 blocks per individual). The 4 paraffin blocks were sectioned at 3 µm, mounted onto glass slides, and stained with hematoxylin and eosin (H&E). In addition to examination by IZVG Pathology Laboratory, these blocks were also submitted to the University of Florida Marine Animal Disease Laboratory, Gainesville, FL, USA.

Transmission electron microscopy

One paraffin block was selected by evaluating an H&E-stained slide for features consistent with megalocytivirus pathology. Heart tissue was selected from this block for TEM evaluation and sent to the Electron Microscopy Laboratory (EML), Department of Medical Pathology and Laboratory Medicine, School of Medicine, University of California at Davis. An area with characteristic megalocytivirus pathology in the heart was removed from the paraffin block and placed in 100% xylene; after clearing overnight, the tissue was rehydrated and processed using a standard protocol (Johannessen 1977) as previously described (Hayat 1989). Ultrathin sections (45 to 60 nm) were post-stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate and examined using a transmission electron microscope at the EML. These samples were also examined with an electron microscope at the University of Florida Electron Microscopy and Bio-imaging Laboratory.

DNA extraction, PCR amplification, and sequencing

At the University of Florida Marine Animal Disease Laboratory, DNA was extracted from 50 µm sections of formalin-fixed, paraffin-embedded tissues using a commercial extraction kit (DNeasy® Blood and Tissue Kit, Qiagen) following the manufacturer's instructions for paraffin-embedded tissues. PCR amplification of the viral full-length major capsid protein (MCP) gene sequence was performed using 14 primer pairs designed from the complete MCP gene sequence of BCIV (which is also known as the *Terapogon kauderni* iridovirus or PkIV; GenBank accession no. AB669096.1) (Table 1).

The 20 µl PCR mixtures consisted of 0.1 µl of Platinum Taq DNA Polymerase (Invitrogen), 2.0 µl of 10× PCR buffer, 0.8 µl of 50 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs, 1.0 µl of 20 µM of forward and reverse primers, 11.7 µl of molecular grade water, and 3 µl of DNA template. The PCR conditions used for all reactions included an initial denaturation of 5 min at 95°C, followed by 50 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. After electrophoresis, bands were gel extracted using a QIAquick gel extraction kit (Qiagen). Purified DNA fragments were then submitted to the University of Florida Interdisciplinary Center for Biotechnology Research for sequencing on ABI 3130 DNA sequencers (Applied Biosystems).

Table 1. Primers used to amplify fragments of the major capsid protein gene sequence from the orbiculate batfish iridovirus (OBIV). Note that amplicon size includes primers

Primer	Orientation	Sequence (5′–3′)	Amplicon size (bp)
MCPF1	Forward	CTGTTGGTCTTGCTGAGTGC	158
BeginR	Reverse	CCGTACAAGTGGGTCTCCAT	
Angelnew1F	Forward	GTTCATCGACATCTCCGCGT	177
Angelmcp1R	Reverse	GCCACCGTGACACTAAACTC	
Angelmcp2fb	Forward	GGGGTGGCGACTACCTCATT	194
Angelmcp2R	Reverse	CCAGGTCGTTAAATGACACCG	
Angelmcp3F	Forward	CAGCTACATTCGCTGGTGCGAC	150
Angelmcp3R	Reverse	GCATGCCAATCATCTTGT	
SmallgapF	Forward	CTGGAACGCCTGCATGAT	124
SmallgapR	Reverse	ATAGTCTGGCCGTTGGTGAT	
MegalofixedF	Forward	ACAAGATGATTGGCATGCG	166
MegalofixedR	Reverse	TTGAAGTGGATGCGCACCT	
Biggap1Fa	Forward	GGCGTTGCCTACTGTGTCTC	163
Big1Ra	Reverse	CAGGGTGACGGTTGATATGG	
Biggap1Fb	Forward	GGACCTGCTCATCAGCCAGAG	165
Angelnew1R	Reverse	CTACGACTAGACTGGGCCA	
Angelnew2F	Forward	CTGACAAGCGAGGAGCGTG	214
Angelnew2R	Reverse	GGGGACTGGCCGCGGTGTAG	
Angelnew3F	Forward	TCACCCACCGCAACGTGC	188
Angelnew3R	Reverse	GGGCGCAAAGTAGTAGG	
Biggap2Fa	Forward	GCTCCACCAGATGGGAGTAG	153
Biggap2Ra	Reverse	GACAGGCGGCCGTAGTTG	
Biggap2Fb	Forward	GGACATGGGCAATATCAACC	132
Biggap2Rb	Reverse	GTGTAGCCGGAGCCGTTG	
Angelnew5F	Forward	GGACAATGCAAAGACCA	145
ISKNVMCPF	Reverse	TTACAGGATAGGGAAGCCTGC	
EndF	Forward	GGTCAAGTTTGAAAACCCGA	121
MCPR4	Reverse	CATAGCTACCAGACACACGG	

BLASTN, molecular data set, sequence alignment, and phylogenetic analysis

Following sequence assembly and removal of primer sequences, general BLASTN searches (www.ncbi.nlm.nih.gov/blast/Blast.cgi) of the full-length viral MCP gene sequence were conducted (Altschul et al. 1997). Megalocytiviral taxa identified by these analyses were combined with taxa from recent comprehensive phylogenetic analyses (Kurita & Nakajima 2012, Waltzek et al. 2012) to build the final data set. Sequence alignments were performed using Mafft 5.8 (Katoh et al. 2005) followed by minor manual adjustments in ClustalW (Thompson et al. 1994). The E-INS-I alignment strategy was used with the following parameters: scoring matrix BLOSUM62, a gap open penalty of 1.53, and an offset value of 0. The final aligned data set was trimmed to the first conserved nucleic acid at the 3′ end of the MCP sequence due to incomplete data being available for certain important taxa. The aligned data set was imported into jModelTest version 0.1.1 (Guindon & Gascuel 2003, Posada 2008) and Akaike's information criterion was used to select a best-fit model of evolution for phylogenetic analysis. Maximum likelihood analyses were conducted using MEGA version 5 (Tamura et al. 2011) with 1000 bootstrap replicates selected for determining node support.

RESULTS

Histopathology

Histopathologic examination revealed multiple areas of necrosis with pyknotic and karyorrhectic cellular debris in the renal hematopoietic tissue (with occasional associated minimal epithelial necrosis) (Fig. 1A), liver, and spleen. Numerous cytomegalic cells containing granular basophilic intracytoplasmic inclusions were commonly observed in the renal interstitium and glomeruli of both specimens (Fig. 1A). Similar cytomegaly with associated inclusions were also observed within the spleen, liver, gill lamellae (Fig. 1B), lamina propria and submucosa of the esophagus, stomach (Fig. 1C), intestine, and heart (Fig. 1D).

Transmission electron microscopy

TEM revealed numerous icosahedral virus particles arranged in paracrystalline arrays consistent with an iridovirus within the cytoplasm of enlarged cardiac myocytes (Fig. 2). The observed viral assembly sites appeared to disrupt the myocyte myofibrils (Fig. 2B). Virus particles were naked with an electron-dense nucleic acid core surrounded by a translucent zone and an outer nucleocapsid layer (Fig. 2C,D). The mean diameter of virus particles, measured from apex to apex, was 158 nm (n = 50, SD = 7.5 nm), and from side to side was 128 nm (n = 50, SD = 5.6 nm).

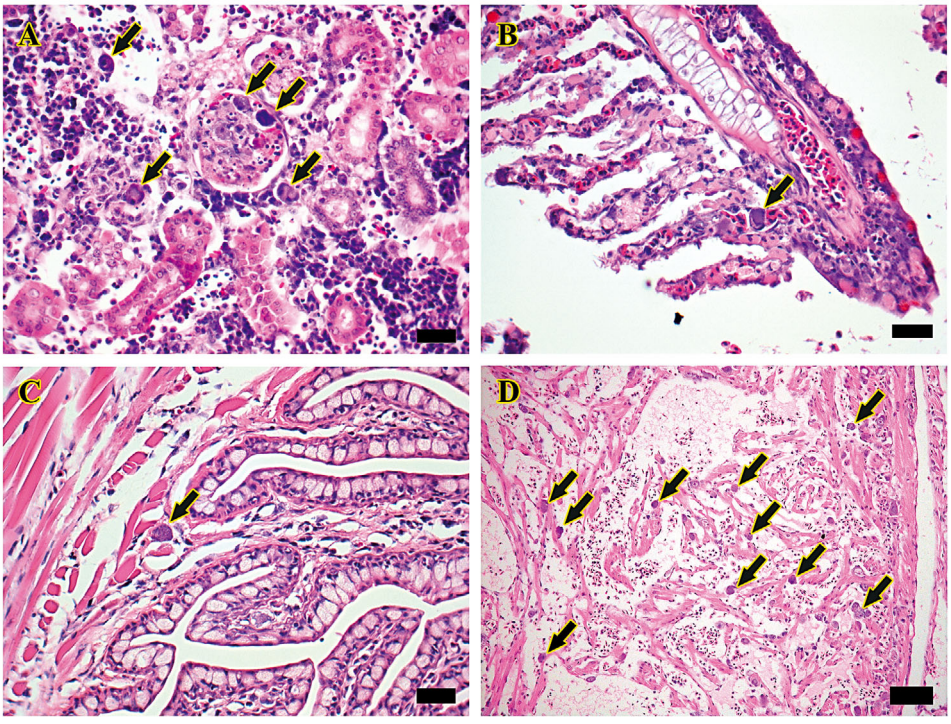

Fig. 1. *Platax orbicularis*. Intracytoplasmic basophilic inclusions (arrows) in infected orbiculate batfish. H&E stain. (A) Interstitium and glomeruli of kidney. Scale bar = 20 µm. (B) Secondary lamellae of gill. Scale bar = 20 µm. (C) Submucosa of stomach. Scale bar = 20 µm. (D) Heart. Scale bar = 50 µm

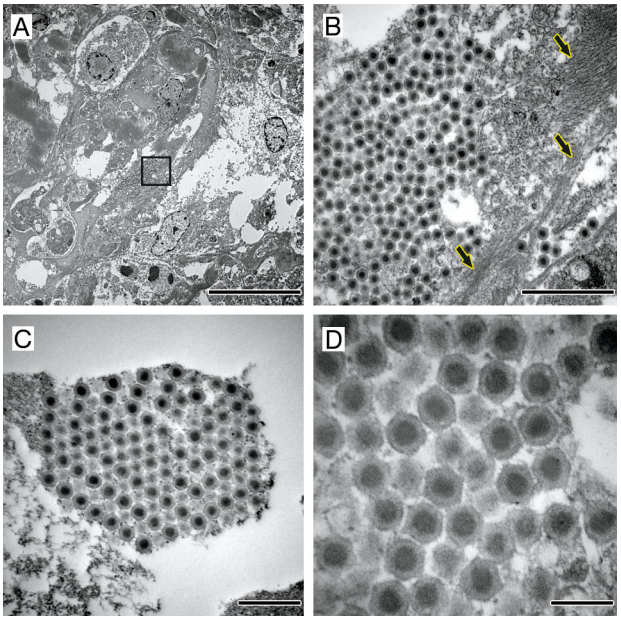

Fig. 2. *Platax orbicularis*. Transmission electron photomicrographs of iridoviral-infected cells in the heart of an orbiculate batfish. (A) Cluster of virus particles in the cytoplasm of an infected myocyte; black rectangle outlines area shown in (B). Scale bar = 10 µm. (B) Numerous virus particles disrupting myocyte myofibrils (arrows). Scale bar = 1 µm. (C) Virions arranged in a paracrystalline array. Scale bar = 500 nm. (D) Higher magnification of (C) revealing the naked icosahedral virus shape and the electron-dense nucleic acid core surrounded by a pale zone and an outer nucleocapsid layer of moderate electron density. Scale bar = 200 nm

Sequencing, BLASTN, molecular data set, and phylogenetic analysis

The sequenced PCR amplicons generated in this study ranged in size from 121 to 214 bp (Table 1) and when assembled resulted in 1470 contiguous bp of the viral genome that contained the full-length MCP sequence (1362 bp; BankIt no. 1592328). The BLASTN search of the viral MCP sequence from the infected batfish revealed highest sequence identity with ISKNV genotype 2 megalocytiviruses, including BCIV (GenBank accession no. AB669096; 100%) and MSGIV (GenBank accession no. HM067835; 99%). The final aligned MCP data set after truncation of the 3′ end of the sequence contained 1357 nucleic acid characters for 90 taxa. jModelTest identified the TrN+G model to be the most suitable model for phylogenetic analyses.

The phylogenetic analysis of the MCP gene supported the recognition of 4 species within the genus *Megalocytivirus* (Fig. 3). The Maximum Likelihood analysis demonstrated with a high level of confidence that the identical virus from orbiculate batfish (hereafter referred to as the orbiculate batfish iridovirus, OBIV) and Banggai cardinalfish (BCIV) is the sister group to MSGIV. These 2 megalocytiviruses form the ISKNV genotype 2 clade (Fig. 3).

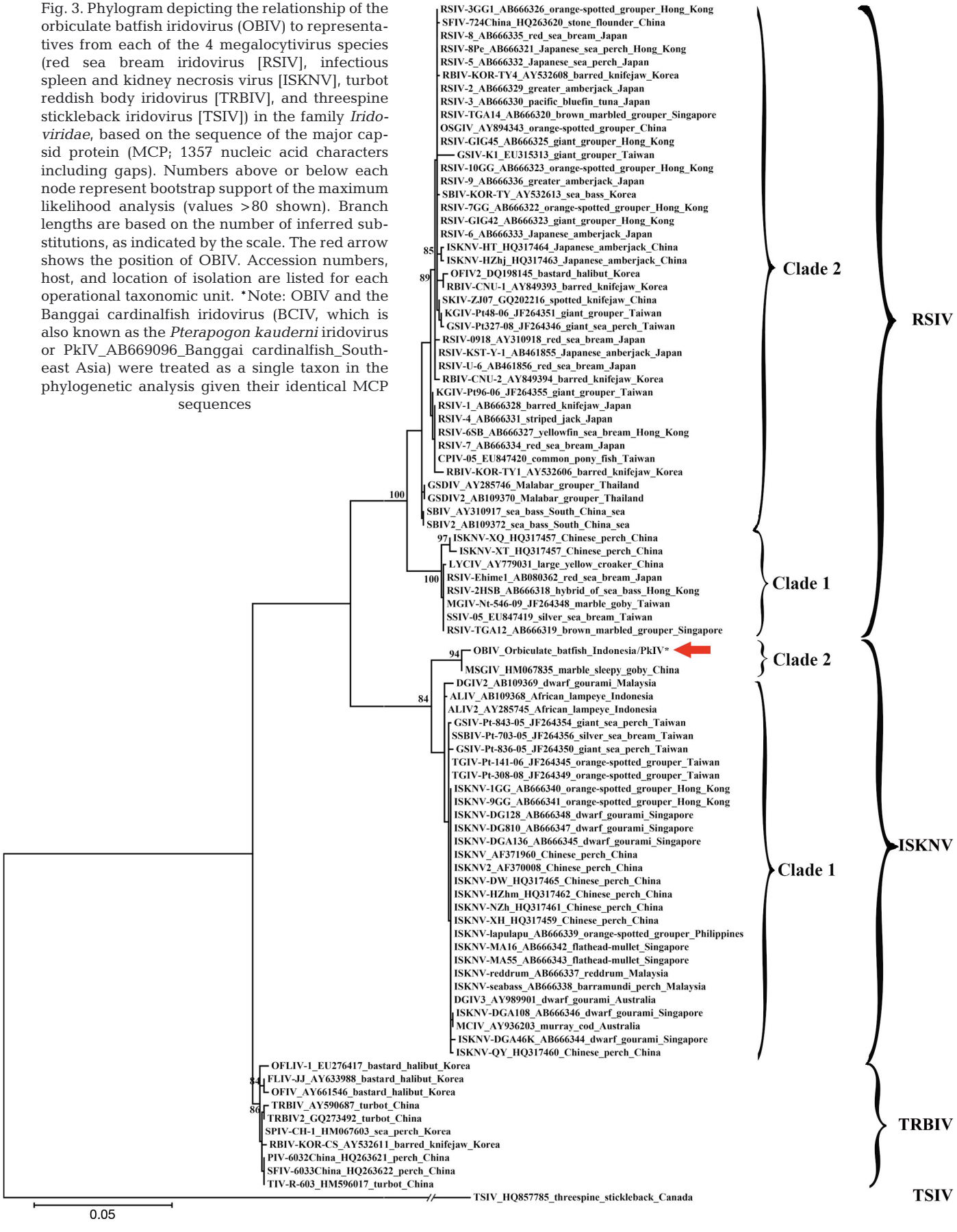

Fig. 3. Phylogram depicting the relationship of the orbiculate batfish iridovirus (OBIV) to representatives from each of the 4 megalocytivirus species (red sea bream iridovirus [RSIV], infectious spleen and kidney necrosis virus [ISKNV], turbot reddish body iridovirus [TRBIV], and threespine stickleback iridovirus [TSIV]) in the family *Iridoviridae*, based on the sequence of the major capsid protein (MCP; 1357 nucleic acid characters including gaps). Numbers above or below each node represent bootstrap support of the maximum likelihood analysis (values >80 shown). Branch lengths are based on the number of inferred substitutions, as indicated by the scale. The red arrow shows the position of OBIV. Accession numbers, host, and location of isolation are listed for each operational taxonomic unit. *Note: OBIV and the Banggai cardinalfish iridovirus (BCIV, which is also known as the *Pterapogon kauderni* iridovirus or PkIV_AB669096_Banggai cardinalfish_Southeast Asia) were treated as a single taxon in the phylogenetic analysis given their identical MCP sequences

DISCUSSION

This study adds to the growing literature on the emerging threat of megalocytiviruses to the international ornamental fish trade (Armstrong & Ferguson 1989, Anderson et al. 1993, Rodger et al. 1997, Sudthongkong et al. 2002, Weber et al. 2009, Kim et al. 2010, Yanong & Waltzek 2010). Here we present the first case of a megalocytivirus outbreak in orbiculate batfish based on histologic, ultrastructural, and genetic evidence. Infected cytomegalic cells displayed basophilic granular intracytoplasmic inclusions in various tissues, similar to descriptions in previous reports, including the report of megalocytivirus infection in Banggai cardinalfish (Gibson-Kueh et al. 2003, Weber et al. 2009, Yanong & Waltzek 2010, Zhang et al. 2011, Waltzek et al. 2012). The size, icosahedral shape, and intracytoplasmic location of virions is also consistent with reports of megalocytivirus infections in tropical freshwater and marine ornamental fishes, including Banggai cardinalfish (Paperna et al. 2001, Sudthongkong et al. 2002, Gibson-Kueh et al. 2003, Weber et al. 2009).

The OBIV full-length MCP sequence was found to be identical to the only other megalocytivirus reported from a marine ornamental fish species, the Banggai cardinalfish iridovirus (Weber et al. 2009). Interestingly, concurrent with the orbiculate batfish mortality event, recently imported wild Banggai cardinalfish that had been kept at the same quarantine facility in the UK also died after delivery to another public aquarium in Denmark. Those fish were found to be positive for BCIV by histopathology and PCR (M. F. Stidworthy & T. B. Waltzek unpubl. data). Taken together with the genetic evidence, these data suggest that OBIV and BCIV are the same virus. This agent may be capable of infecting other species, similar to what has been reported for other megalocytivirus species (Kurita & Nakajima 2012).

It cannot be determined whether the imported wild orbiculate batfish and Banggai cardinalfish spread the virus to each other following importation into the UK or whether these species had already acquired the virus in the wild or at the export facilities. Although the Banggai cardinalfish is restricted to a small region in Indonesia known as the Banggai archipelago, the orbiculate batfish occurs sympatrically, and both species are sometimes cultured on the same Indonesian ornamental fish farms for sale in international ornamental fish markets (T. B. Waltzek pers. obs.). Future surveillance efforts are needed to determine where marine ornamental fishes become infected with megalocytiviruses as well as to assess the overall impact on international trade (e.g. frequency of epizootics and number of susceptible species).

Acknowledgements. M. Potin, T. Defonseca, R. James, G. Adamson, J. F. Wellehan, and L. Archer provided technical assistance. We thank A. S. Kane and J. E. Hill for their constructive criticism of the manuscript.

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Editorial responsibility: V. Gregory Chinchar, Jackson, Mississippi, USA

Submitted: January 2, 2013; Accepted: March 4, 2013
Proofs received from author(s): June 11, 2013