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# MOLECULAR CHARACTERISATION OF GROUPER IRIDOVIRUS ISOLATES FROM PENINSULAR MALAYSIA

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## SUMMARY

Grouper Iriovirus (GIV) is one of the most devastating viral diseases of marine and cultured groupers worldwide. In the current study, 5 presumptive Malaysian GIV isolates were characterised through PCR amplification of the major capsid protein (MCP) gene and phylogenetic analysis of the sequences. The sequences from the five GIV isolates showed 100% homology with each other and a close relationship with grouper iridovirus isolate (GIV_Tn_352), which was clustered in group 1 together with King grouper iridovirus isolate (KGIV_Cy_346), Singapore grouper iridovirus (SGIV), and Crimson snapper iridovirus isolate (CSIV). The phylogenetic tree also showed different degree of relatedness with other Ranavirus strains which were obtained from the blast of GIV MCP gene in the NCBI database. This study confirmed the GIV isolates from Malaysia are related to other isolates that were reported previously.

*Keywords: Grouper, Iridovirus, characterization, homology, PCR*

## INTRODUCTION

Several countries have over the past two decades reported iridoviruses in disease epizootics of amphibians and fish. This is of concern as the international trade in fish has been responsible for epidemics in Asia. Piscine iridoviruses, found in many countries in Asia, have been grouped into three genera: *Lymphocystivius, Ranavirus,* and *Megalocytivirus* (Wang *et al.,* 2007; Hossain *et al.,* 2008). Based on our recent study, evidence of natural Grouper Iridovirus (GIV) infection was confirmed in Malaysian cultured fish through histopathological and molecular detection (Hazeri *et al*., 2016). According to molecular genetics and microscopic characteristics of GIV in grouper fish, the virus was identified to be a member of the genus Ranavirus (Murali *et al.,* 2002; Tsai *et al.,* 2005). The most important gene used in analysing the genetic relationships among the Iridoviridae family members is the major capsid protein (MCP) gene, which is a structural protein with highly conserved domains (Do *et al.,* 2005; Go *et al.,* 2006) that encapsulate the most detailed evolutionary information of the virus.

Since there is no information on the molecular characterisation of GIV in Peninsular Malaysia, it is paramount to investigate the genetic relatedness of Malaysian isolates of GIV with other reported ones worldwide. This study was thus conceived to investigate the genetic variety of GIV in Malaysia through characterisation of complete MCP genes from isolates obtained from cases of natural GIV infection from cultured grouper fish farms in Peninsular Malaysia.

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## MATERIALS AND METHODS

### *Sample preparation for PCR assay*

Five GIV isolates collected from two grouper species i.e. Tiger grouper hybrid (*Epinephelus sp.*) and Coral trout (*Plectropomus leopardus*) as reported earlier were used for this study (Hazeri *et al.,* 2016). The virus isolates were cultured in BF-2 cells (ATCC CRL-2050) at 25-28ºC with Eagles' modified essential medium containing 10% fetal bovine serum and infected cells were repeatedly frozen in -70°C and thawed three times. Following clarification by low-speed centrifugation (Beckman, Germany) at 3,000 rpm for 15 min at 4°C, the resultant supernatant fluid was harvested. The supernatant was further clarified by centrifugation at 10,000 rpm for 30 min at 4°C. The collected supernatant was then filtered through a 0.45 µm membrane filter and kept at -70°C prior to use.

### *DNA Extraction, Primer design and PCR*

DNA was extracted using protocol described previously (Hazeri *et al*., 2016). Primers for the gene encoding MCP were designed for conventional PCR using nucleotide sequence of GIV (AY666015). One set of primer (RM1-F 5'-CTCCCGTTGCCGTTCTTT-3'; RM1-R5'-CCTGAAGCGACCTCAGTTTAAT-3') was designed using the IDTDNA online software (*https://sg.idtdna.com/site)* and utilised for the amplification of 1,392 base pairs from the GIV genomes of the isolates. The PCR amplification was carried out as reported by Huang (2011) with slight modification as follows; a final reaction volume of 25 µL was used comprising of 2 µL of template DNA, 5 µL of 5x green GoTaq flexi buffer (Promega, USA), 2.5 µL of 25 mM magnesium chloride (Promega, USA), 0.5 µL of 10mMdNTPs (Promega, USA), 0.25 µL of 5 U $\mu L^{-1}$ *Taq*

polymerase (Promega, USA), 2 µL (10 µM) of forward and reverse primer, and 10.75 µL of sterile distilled water. The reaction mixture was ran in a thermalcycler (Bio-Rad, USA) at 95°C for 5 min and then for 33-35 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 50 s and extension at 72°C for 60 s. A final extension at 72°C for 7 min was done. The PCR products were analysed on 2% agarose gel containing ethidium bromide (Invitrogen, USA) and visualised using Bio Imaging System (Syngene, UK).

*PCR Product Purification, Plasmid Cloning and Sequencing*

The positive GIV-PCR products were then subjected to purification with QIA quick PCR purification kit (Qiagen, Germany) and cloned with PCR Cloning Plus (Qiagen, Germany) following the manufacturer's instructions. White colonies were chosen for recombinant plasmid and purified with QIAprep Spin Miniprep (Qiagen, Germany). Sets of primers were employed using PCR to verify the success of the transformation. Following purification the plasmids were then subjected to commercial sequencing (First BASE Laboratories, Malaysia) employing Sanger's method. The sequence assembly and alignments were carried out with Bio Edit Sequence Alignment Editor (V 7.0.5.2). A comparison was then made of the DNA sequences with the GenBank database employing the basic local alignment search tool (BLAST). Overall, 19 MCP gene sequences were utilised for phylogenetic analysis, these include the 5 GIV isolates from this study, 12 *Ranavirus* isolates and two members of genus *Megalocytivirus* as reference nucleotide sequences obtained from the GenBank database. Alignment of the DNA sequences was done using MEGA version 7.0, and the phylogenetic relationship of MCP gene sequences was established utilising the neighbour joining method with the Tamura Nei algorithm (Tamura *et al.*, 2011).

## RESULTS

*Amplification of GIV DNA*

The representative amplicons obtained by PCR from the 5 GIV isolates is shown in Figure1. Samples were positive with GIV primers amplification and sequence similarity search confirmed the isolates were GIV. The isolates were thus named GIV (Malaysia GIV) and deposited in the GenBank under accession numbers (Seq1: KX228764, Seq2: KX228765, Seq3: KX228766, Seq4: KX228767 and Seq5: KX228768). The major capsid protein gene was successfully amplified with a resultant product size of 1,392 bp.

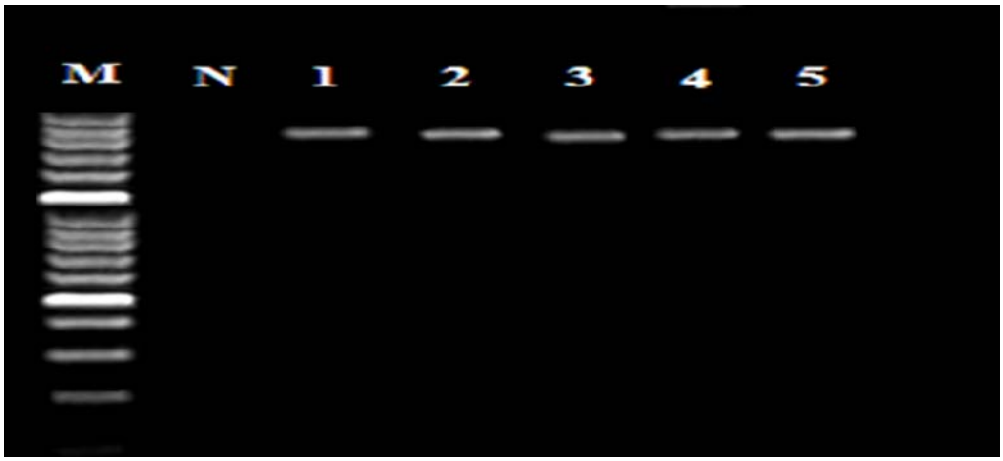


**Figure 0. PCR results of 5 GIV isolates cultured in BF-2 cells (lanes 1, 2, 3, 4 and 5), on agarose gel showing positive amplicons of MCP gene (1,392 bp)**

*Nucleotide sequence and phylogenetic analysis*

The 5 GIV isolates reported here were identical to the reference GIV MCP DNA sequence (1392bp). The sequences for the five GIV isolates had 100% homology over the 1,392 base pairs and therefore highly identical based on the MCP. The MCP sequences from the GIV isolates obtained in this study were compared with the MCP sequences from *Ranavirus* (GenBank) which included the gene and the proximal flanking regions. Alignment of the sequences revealed 98- 99% homology with 5 King grouper iridovirus isolates (KGIVTn 337), King grouper iridovirus isolate (KGIV_Cy_346), Singapore grouper iridovirus (SGIV), Crimson snapper iridovirus isolate (CSIV), and Grouper iridovirus isolate (GIV_Tn_352) (Table 2). MCP sequence of GIV had 97% similarity with Grouper iridovirus isolate (GIV_Pt_403), Largemouth bass virus isolate (LMBIV) and Giant seaperch iridovirus isolate (GSIV), and 96% similarity with Grouper iridovirus (GIV complete genome), King grouper iridovirus isolate (KGIV_Pt_1691), King grouper iridovirus isolate (KGIV_Pt_1348) and King grouper iridovirus isolate (KGIV_Pt_1241). Query cover between GIV and all sequences above is 99%, which also revealed a high degree of homology between these groups of viruses (Table 1).

**Table 1. Nucleotide Similarity percentage of GIV-MCP with 12 other isolates of ranavirus obtained from the NCBI database**

| GenBank Fish Ranavirus isolates | Accession No. | Nucleotide similarity percentage | Country |
|---|---|---|---|
| KGIVTn 337 | JF264360.1 | 99% | Taiwan |
| KGIV_Cy_346 | JF264366.1 | 99% | Taiwan |
| SGIV | AY521625.1 | 99% | Singapore |
| CSIV | JF264357.1 | 99% | Taiwan |
| GIV_Tn_352 | JF264358.1 | 99% | Taiwan |
| GIV_Pt_403 | JF264365.1 | 97% | Taiwan |
| LMBIV | JF264364.1 | 97% | Taiwan |
| GSIV | JF264362.1 | 97% | Taiwan |
| GIV complete genome | AY666015.1 | 96% | Taiwan |
| KGIV_Pt_1691 | JF264367.1 | 96% | Taiwan |
| KGIV_Pt_1348 | JF264363.1 | 96% | Taiwan |
| KGIV_Pt_1241 | JF264359.1 | 96% | Taiwan |

Based on the phylogenetic tree generated (Figure 2), sequences from the 5 GIV isolates showed close relationship with GIV isolate (GIV_Tn_352), and is clustered in group 1 together with King grouper iridovirus isolate (KGIV_Cy_346), Singapore grouper iridovirus (SGIV), and Crimson snapper iridovirus isolate (CSIV). The tree showed different degree of relatedness with other Ranavirus strains which were obtained from the blast of GIV MCP gene in the NCBI. The nucleotide sequence identity between genotype 1 and 2 is below 97% and between genotype 1 and 3 is about 94% (Table 2).The farthest group had strains belonging to *Megalocytivirus*, Infectious spleen and kidney necrosis virus (ISKNV) from China and Red sea bream iridovirus (RSIV) from Japan.

Pairwise comparison methods were employed in clarifying how fish hosts and virus strains were related in order to establish the maximum identity of the MCP gene from the five isolates (Table 2).

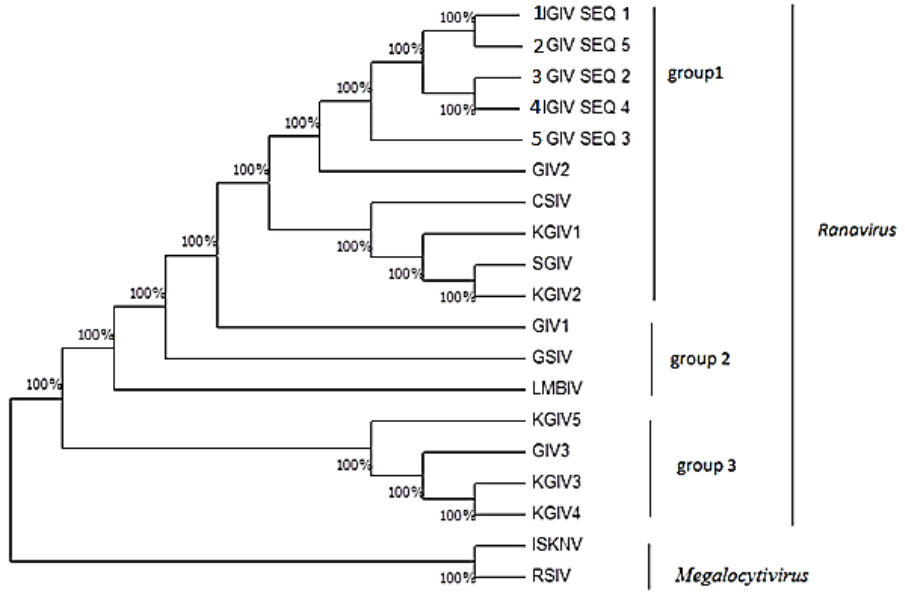


**Figure 2: Phylogenetic tree deduced from the GIV-MCP gene was compared with 12 Ranavirus and 2 Megalocytivirus.**

The GIV was closely related to Grouper iridovirus isolate (GIV_Tn_352, JF264358.1,GIV2) NO. 1 , 2 , 3 ,4 and 5: The GIV isolated in current study (Seq1 : KX228764, Seq2: KX228765, Seq3: KX228766, Seq4: KX228767 and Seq5: KX228768), GIV1: Grouper iridovirus isolate (GIV_Pt_403, JF264365.1, Taiwan), GIV2: Grouper iridovirus isolate (GIV_Tn_352, JF264358.1, Taiwan), GIV3: Grouper iridovirus (GIV complete genome, AY666015.1, Taiwan), KGIV1: King grouper iridovirus isolate (KGIVTn 337, JF264360.1, Taiwan), KGIV2: King grouper iridovirus isolate (KGIV_Cy_346, JF264366.1, Taiwan), KGIV3: King grouper iridovirus isolate (KGIV_Pt_1691, JF264367.1, Taiwan), KGIV4: King grouper iridovirus isolate (KGIV_Pt_1348, JF264363.1, Taiwan), KGIV5: King grouper iridovirus isolate (KGIV_Pt_1241, JF264359.1, Taiwan), ISKNV: Infectious spleen and kidney necrosis virus (ISKNV, AF370008, China), RSIV: Red sea bream iridovirus (RSIV, AY310918, Japan), Singapore grouper iridovirus (SGIV, AY521625.1,Singapore), Crimson snapper iridovirus isolate (CSIV, JF264357.1, Taiwan), Largemouth bass virus isolate (LMBIV, JF264364.1, Taiwan) and Giant seaperch iridovirus isolate (GSIV, JF264362.1, Taiwan)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 99.28 | 99.36 | 98.72 | 98.53 | 99.7 | 98.78 | 98.13 | 98.54 | 98.76 | 97.97 | 97.7 | 97.15 | 96.53 | 96.15 | 96.15 | 96.07 | 57.75 | 156.88 |
| 2 | | 100 | 99.28 | 98.10 | 98.28 | 99.7 | 99.7 | 98.07 | 98.03 | 98.10 | 97.63 | 97.72 | 97.37 | 96.68 | 95.88 | 95.53 | 95.45 | 56.97 | 56.05 |
| 3 | | | 100 | 99.36 | 99.36 | 98.25 | 98.07 | 98.10 | 98.25 | 98.51 | 97.75 | 97.68 | 97.52 | 95.88 | 96.86 | 95.60 | 96.81 | 57.80 | 56.88 |
| 4 | | | | 100 | 99.10 | 99.7 | 98.72 | 99.26 | 98.08 | 98.72 | 97.72 | 97.84 | 97.04 | 96.96 | 96.81 | 96.03 | 96.11 | 56.73 | 56.83 |
| 5 | | | | | 100 | 99.6 | 98.83 | 99.19 | 98.84 | 99.16 | 97.12 | 97.35 | 97.25 | 96.13 | 96.83 | 96.68 | 95.1 | 57.07 | 57.83 |
| 6 | | | | | | 100 | 99.6 | 98.03 | 98.24 | 99.10 | 97.72 | 97.27 | 97.72 | 96.86 | 95.66 | 94.29 | 95.50 | 56.77 | 57.61 |
| 7 | | | | | | | 100 | 99.15 | 99.85 | 98.48 | 97.57 | 97.68 | 97.02 | 96.24 | 96.35 | 95.95 | 95.50 | 56.29 | 57.13 |
| 8 | | | | | | | | 100 | 98.96 | 99.17 | 97.68 | 97.8 | 97.07 | 96.15 | 96.98 | 95.8 | 95.38 | 56.48 | 57.30 |
| 9 | | | | | | | | | 100 | 98.18 | 97.72 | 97.65 | 97.28 | 95.46 | 96.18 | 96.81 | 96.57 | 56.26 | 56.96 |
| 10 | | | | | | | | | | 100 | 97.07 | 97.47 | 97.72 | 96.50 | 96.88 | 96.14 | 95.95 | 56.46 | 56.12 |
| 11 | | | | | | | | | | | 100 | 97.48 | 97.82 | 96.81 | 96.33 | 96.14 | 96.57 | 56.29 | 55.98 |
| 12 | | | | | | | | | | | | 100 | 97.28 | 96.1 | 95.1 | 96.81 | 96.03 | 56.37 | 56.48 |
| 13 | | | | | | | | | | | | | 100 | 96.36 | 96.18 | 95.87 | 96.96 | 56.88 | 56.12 |
| 14 | | | | | | | | | | | | | | 100 | 96.07 | 95.71 | 96.11 | 56.88 | 56.96 |
| 15 | | | | | | | | | | | | | | | 100 | 96.0 | 96.57 | 55.98 | 56.46 |
| 16 | | | | | | | | | | | | | | | | 100 | 96.03 | 57.07 | 56.29 |
| 17 | | | | | | | | | | | | | | | | | 100 | 56.83 | 57.30 |
| 18 | | | | | | | | | | | | | | | | | | 100 | 57.61 |
| 19 | | | | | | | | | | | | | | | | | | | 100 |

**0 2. Percentage of nucleotide sequence identity matrix of the MCP gene between strains of GIV detected in present study and 12 reference viruses from genus Ranavirus and 2 reference viruses from genus Megalocytivirus retrieved from GenBank database.**

1, 2, 3, 4 and 5: GIV isolated in current study (Seq1: KX228764, Seq2: KX228765, Seq3: KX228766, Seq4: KX228767 and Seq5: KX228768), 6: Grouper iridovirus isolate (GIV_Tn_352, JF264358.1, Taiwan), 7: King grouper iridovirus isolate (KGIVTn 337, JF264360.1, Taiwan), 8: Singapore grouper iridovirus (SGIV, AY521625.1, Singapore), 9: King grouper iridovirus isolate (KGIV_Cy_346, JF264366.1, Taiwan), 10: Crimson snapper iridovirus isolate (CSIV, JF264357.1 , Taiwan), 11: Grouper iridovirus isolate (GIV_Pt_403, JF264365.1, Taiwan), 12: Largemouth bass virus isolate (LMBIV, JF264364.1, Taiwan), 13: Giant seaperch iridovirus isolate (GSIV,JF264362.1, Taiwan), 14: Grouper iridovirus (GIV complete genome, AY666015.1, Taiwan), 15: King grouper iridovirus isolate (KGIV_Pt_1691, JF264367.1, Taiwan), 16: King grouper iridovirus isolate (KGIV_Pt_1348, JF264363.1, Taiwan), 17: King grouper iridovirus isolate (KGIV_Pt_1241, JF264359.1, Taiwan), 18: Infectious spleen and kidney necrosis virus (ISKNV , AF370008, China), 19: Red sea bream iridovirus (RSIV, AY310918, Japan)

## DISCUSSION

In this study, conventional PCR amplification yielded positive results for GIV MCP gene. The isolated iridoviruses from grouper fish (GIV) can be grouped as members of the Ranavirus according to the sequence analysis and phylogenetic analysis. In earlier studies, host species and geographical range were the criteria used in classifying the Ranaviruses because they were mainly found in distinctive hosts and geographical regions (Hyatt *et al*., 2000; Chinchar, 2002; Jancovich *et al.*, 2003; Williams *et al.,* 2005). Traditional PCR has been utilised for detection of ranavirus infection in fresh or fixed tissue specimens through surveillance studies and mortality events. Cultured viruses have also been identified using this technique (Miller *et al.,* 2007; *Gray et al.*, 2009; Meng*et al.,* 2014). The results of this study reinforces that the strain of virus in the current study, even though obtained from different farms were highly similar, suggesting that the virus circulating in the fish farms were similar and are ranaviruses.

Phylogenetic mapping of banked DNA sequences (GenBank) that represent isolates worldwide is used to show significances in sequence identities, which has been beneficially used to design primers for the purpose of detecting ranaviruses through PCR. Additionally, sequence polymorphisms in the MCP gene can be useful for distinguishing isolates to the genus level (e.g., GIV, SGIV). Typical PCR for the purpose of detecting ranaviruses in order to subtype them are found in the *OIE Diagnostic Manual for Aquatic Animal Diseases* (OIE, 2012). According to the results of this study, the primer setdesigned had detected GIV with a rather high sensitivity.

DNA sequencing of traditional PCR products for purposes of confirming positive findings may not always be needed, particularly in endemic regions. On the other hand, the MCP gene is a suitable target for the inference of phylogenetic relationship because it is conserved and diverse enough to distinguish closely-related isolates (Tidona *et al*., 1998). There is the possibility of genotyping with the use of traditional PCR and DNA sequencing for genes like the intergenic variable region (Jancovich *et al*., 2005; Weir *et al.,* 2012). Based on the phylogenetic tree profiles from the MCP gene, the genotypes of GIV isolates have a close relationship with KGIV, SGIV, CSIV, LMBIV and GIV. It seems that the virus has shown gradual adaptation and evolved into the present local strains. Therefore, in this study, the sequence of the MCP gene was utilised to confirm the phylogenetic relationship among the GIV isolates detected in Peninsular Malaysia and reference sequences derived from the GenBank database.

The current phylogenetic analysis indicated 3 genotypes in genus *Ranavirus*, and all the GIV isolates that were identified from grouper species namely tiger grouper hybrid (*Epinephelus* sp.) and coral trout (*Plectropomus leopardus*) from Peninsular Malaysia were assigned to genotype 1. Numerous researches have provided evaluations of how the epidemic strains, geographic distribution, and host susceptibility of members of the Ranavirus are genetically related. In Malaysia, ranaviruses were found in the states of Selangor and Kedah, mostly in marine fish (Tiger grouper hybrid and Coral trout). Iridovirus infection has been reported in Sabah, Malaysia with an incidence of 33.02% using both MCP and ATpase genes (Razak *et al.,* 2014). However, there is no report from Peninsular Malaysia of grouper iridovirus. Hence, the high variability of the MCP gene could prove to be very useful in studying virus strain evolution, classification, and taxonomic differentiation. Continuous observation and monitoring of the iridovirus genotypes using a global database may enhance the understanding of local viral genotypechanges and how they relate to worldwide epidemiology. This information will facilitate the diagnosis and management of the disease including research into development of vaccines and potential cures.

## CONCLUSION

In the current study, sequence analysis of MCP gene confirmed that the Malaysian isolates of GIV were similar to each other. The phylogenetic analysis revealed that all the GIV isolates identified in Peninsular Malaysia were categorised in Group 1 along with reference fish ranavirus. This baseline information is essential to mitigate the spread of this disease in the country.

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## ETHICAL CONSIDERATION

Grouper fish used for this study is not an endangered or extinct species, thus there was no provision for special approval. However, agreed consent was given by the concerned fish farmers to conduct the research using moribund fish from their farms.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## AUTHORS CONTRIBUTION

All authors contributed equally to this work and are responsible for the data presented herein.

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