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1 **Development of a SYBR Green quantitative PCR assay for detection of**

2 ***Lates calcarifer herpesvirus* (LCHV) in farmed barramundi**

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13

14 **Running head**: qPCR detection of LCHV

15

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19 **Highlights**

20 - This study reported a new SYBR Green qPCR method for detection of LCHV
21 - The qPCR method had detection limit of 10 copies per µl plasmid DNA template when
22 spiked with genomic DNA from the host
23 - The aforementioned method is highly specific to LCHV
24 - Validation with clinical samples revealed that LCHV could be detected from multiple
25 organs with fin and brain the best organs for qPCR detection

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26 **ABSTRACT**

27 *Lates calcarifer* herpes virus (LCHV) is a new virus of farmed barramundi in Southeast Asia.
28 However, a rapid detection method is yet to be available for LCHV. This study, therefore,
29 aimed to develop a rapid quantitative PCR (qPCR) detection method for LCHV and made it
30 timely available to public for disease diagnostics and surveillance in barramundi farming
31 countries. A newly designed primer set targeting a 93-bp fragment of the LCHV putative
32 major envelope protein encoding gene (*MEP*) was used for developing and optimizing a
33 SYBR Green based qPCR assay. The established protocol could detect as low as 10 viral
34 copies per µl of DNA template in a reaction containing spiked host DNA. No cross-
35 amplification with genomic DNA extracted from host as well as common aquatic pathogens
36 (12 bacteria and 3 viruses) were observed. Validation test of the method with clinical samples
37 revealed that the virus was detected in multiple organs of the clinically sick fish but not in the
38 healthy fish. We thus recommend that barramundi farming countries should promptly initiate
39 active surveillance for LCHV in order to understand their circulation for preventing possibly
40 negative impact to the industry.

41 **Keywords:** detection, *Lates calcarifer* herpes virus (LCHV), qPCR

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42 **INTRODUCTION**

43 Barramundi (*Lates calcarifer*) or Asian sea bass is one of the economically important finfish
44 species in Asia-Pacific which has been farmed in a wide range of salinity in either open cage
45 systems or earthen ponds (Jerry et al., 2014). Barramundi, like other intensively farmed fish,
46 is susceptible to various infectious pathogens and often subject to serious outbreaks and
47 economic losses (Dong et al., 2017a, b; Jerry et al., 2014; Ransangan et al., 2010; Toranzo et
48 al., 2005). In recent years, three newly emerging viruses have been reported in farmed
49 barramundi in Asia-Pacific, including scale drop disease virus (SDDV) (Gibson-Kueh et al.,
50 2012; de Groof et al., 2015), *Lates calcarifer* herpes virus (LCHV) (Chang et al., 2017) and
51 *Lates calcarifer* birnavirus (LCBV) (Chen et al., 2019). Both SDDV and LCHV were
52 discovered from disease outbreaks where the fish showed clinical symptoms of "scale drop"
53 and laboratory infections with the cultivated virus from cell culture resulted in up to 60% and
54 77% cumulative mortality, respectively (de Groof et al., 2015; Chang et al., 2017). By
55 contrast, LCBV did not induce mortality in the controlled laboratory trial (Chen et al., 2019).

56 LCHV discovered by Chang et al. (2017) is a novel member of the family *Alloherpesviridae*,
57 which is genetically most similar to *Ictalurid herpesvirus* 1 (<60% nucleotide identity), a
58 pathogenic virus of channel catfish. LCHV is an enveloped virus with diameter of
59 approximately 100 nm, and genome size of ~130 kb while other members of
60 *Alloherpesviridae* are between 150-250 nm in diameter and 100-250 kb in genome size
61 (Hanson et al., 2011; Chang et al., 2017).

62 Both SDDV and LCHV infections cause similar scale drop disease-like gross signs which are
63 clinically indifferentiable. Therefore, molecular detection methods are required for diagnostic
64 and screening purposes. Several DNA-based detection methods for SDDV have been freely
65 available such as single PCR (Senapin et al., 2019), semi-nested PCR (Charoenwai et al.,

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66 2019), loop-mediated isothermal amplification (LAMP) (Dangtip et al., 2019), probe-based
67 qPCR (de Groof et al., 2015), and SYBR Green-based qPCR (Sriisan et al., 2020). The latter
68 one is the most sensitive method with a detection limit of 2 copies of DNA template per
69 reaction. In case of LCHV, following discovery of the virus, several primer sets for detection
70 purpose were published in a patent (Chang et al., 2017), the use of these methods thus might
71 be conditionally limited. According to requests from private sector, this study, therefore,
72 developed a new, sensitive qPCR detection method for rapid diagnostics of LCHV and made
73 it available to promote active surveillance for preventing wide-spread of this pathogen.

74

75 **MATERIALS AND METHODS**

76 **Fish samples and DNA extraction**

77 In 2019, there were 3 batches of barramundi samples subjected to testing for LCHV in our
78 laboratory. Batch 1 comprised of adult fish (n = 5) in which 4 of them exhibited scale drop
79 clinical signs while one fish had healthy looking appearance. Eight different tissue types
80 (liver, kidney, spleen, gills, fin, brain, eyes and muscle) from each fish were dissected and
81 individually preserved in 95% ethanol. Batch 2 comprised of apparently healthy barramundi
82 fry that were ethanol-preserved. Three whole fry were pooled and considered as one sample
83 for the test (n = 5 pools). Batch 3 (n = 10) comprised of ethanol-preserved spleen samples
84 collected from 5 apparently healthy juvenile fish and 5 clinically sick fish showing scale drop
85 disease-like symptoms. Approximately 5 mg tissue was subjected to DNA extraction using
86 conventional sodium dodecyl sulfate/proteinase K containing lysis solution followed by
87 phenol/chloroform extraction and ethanol precipitation. The obtained DNA pellet was
88 resuspended in sterile distilled water and quantified using spectrophotometry at OD 260 and
89 280 nm.

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90 **Primer design and PCR conditions**

91 LCHV primers were designed to target a 93 bp partial fragment of a putative major envelop
92 protein (*MEP*) gene of the virus. Forward primer LCHV-MEP93-qF: 5'-
93 GTACTTCATCGCCTACGGAGC-3' and reverse primer LCHV-MEP93-qR: 5'-
94 TACGTGTGCTTGAGGAGGTC-3' were synthesized from Bio Basic, Canada. Gradient
95 PCR was firstly conducted to find an optimal annealing temperature (Ta) using Ta ranging
96 from 58 to 65 ºC. The reaction mixture of 20 µL contained 200 ng of DNA extracted from fin
97 of LCHV-infected fish, 1x iTaq Universal SYBR Green SuperMix (Bio-Rad Cat.no. 172-
98 5121) and 200 nM of each primer. The PCR amplification conditions were initial
99 denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 10 s and annealing at 58-
100 65 ºC for 30 s (Bio-Rad CFX Connect Real-Time PCR) followed by melt peak analysis.
101 Finally, Ta of 61°C was selected and the same thermocycling conditions were used
102 throughout the study.

103 **Sensitivity, qPCR efficiency, and specificity assays**

104 Positive control plasmid namely pMEP93 was constructed for use in diagnostic sensitivity
105 test. This was done by cloning the 93-bp *MEP* amplified fragment obtained above into pGEM
106 T-easy vector (Promega) and transforming into *Escherichia coli* XL-1 blue. After colony
107 PCR verification of potential correct clones, one recombinant clone was sent for DNA
108 sequencing at Macrogen (South Korea). Copy number of pMEP93 was calculated based on
109 plasmid size and concentration at https://cels.uri.edu/gsc/cndna.html and the pMEP93 was 10
110 fold-serially diluted from 10^7 to 1 copies/µl. Plasmid dilutions (2 µl) were then used as
111 template in qPCR conditions described above. To mimic a real test, each reaction also
112 contained spiked 100 ng DNA extracted from a healthy barramundi. Control reaction without
113 pMEP93 was used as a negative control. Analytical sensitivity experiment was conducted in

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114 3 replicates within the same run. Standard curve was then automatically generated from
115 quantification cycle (Cq) values being plotted versus $\log_{10}$ pMEP93 quantity. Formula for
116 copy number calculation, coefficient of correlation (R^2), and amplification efficiency (E)
117 values were also provided by the Bio-Rad Maestro Software.

118 The optimized qPCR protocol was subsequently used to test for specificity against extracted
119 genomic DNA from i) clinically healthy fish, ii) from 12 common aquatic bacterial species,
120 and iii) from fish samples infected with either infectious spleen and kidney necrosis virus
121 (ISKNV), nervous necrosis virus (NNV), or scale drop disease virus (SDDV). Sample
122 sources and preparation were previously described (Charoenwai et al., 2019; Sriisan et al.,
123 2020). DNA extracted from fin of LCHV-infected fish was used as positive control. No
124 template control was used as negative reaction. Specificity test was performed in 2 replicates
125 by 2 qPCR runs.

126 **LCHV detection in field samples**

127 The newly developed qPCR was used to detect and quantify LCHV loads in the barramundi
128 DNA samples prepared from 3 fish batches. 200 ng DNA template was used in each qPCR
129 reaction. The obtained Cq was used to calculate viral copy numbers in the samples using the
130 equation, copy number = $10^{(Cq - Intercept)/Slope}$ i.e. $10^{(Cq - 41.34))/-3.539}$ derived from the stand curve
131 described above. Comparative evaluation of the viral loads in different fish tissue types was
132 performed using samples from batch 1.

133

134 **RESULTS**

135 **SYBR Green based LCHV qPCR**

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136 The LCHV qPCR protocol developed in this study had a detection limit of 10 copies/µl
137 template i.e. 20 copies/reaction. Mean Cq ± SD values of the detection limit were 37.91 ±
138 0.33 (**Fig. 1a**). In other words, samples with Cq ≤ 37.91 ± 0.33 were considered as LCHV
139 positive tests. The amplified products yielded uniform melting temperatures (Tm) at 84.0°C
140 (**Fig. 1b**), indicating that the primers and the condition assayed were specific. The 93-bp
141 amplicon had a relatively high Tm due to its 58% GC content of the sequence. Note that there
142 was 1 nucleotide difference (**Supplemental Fig. 1**) between the target sequence in this study
143 and that from the previous data (Chang et al., 2017). Based on the standard curve shown in
144 **Fig. 1c**, the performance of the newly developed qPCR was high determined by its
145 amplification efficiency (E) of 91.7% with R^2 of 0.995. When evaluated the protocol
146 specificity, the LCHV qPCR was demonstrated to be highly specific because it only detected
147 LCHV infected sample but not DNA extracted from 3 other viruses, 12 bacteria, or clinically
148 healthy fish tested. Data from one of the two replicates is shown in **Fig. 2**.

149 **LCHV detection in fish samples**

150 Tissue tropism of LCHV was revealed using the sample batch 1. Among all 8 tissues (liver,
151 kidney, spleen, gills, fin, brain, eyes and muscle) tested from 4 diseased barramundi, the
152 qPCR assay detected LCHV DNA at variable loads in 3-7 tissue types of each fish but not in
153 the kidney samples (**Table 1**). There was only 1 in 4 liver sample which tested positive for
154 LCHV with low viral loads (32 copies/200 ng DNA). DNA extracted from the fin, gills and
155 muscle had averagely higher LCHV loads (24-597 copies/200 ng DNA) when compared to
156 that of the brain, eyes, spleen and liver (13.7-184 copies/200 ng DNA). LCHV was not
157 detected in any of the 8 tissue types of a clinically healthy fish from the same batch (**Table**
158 **1**).

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159 The established qPCR was also applied to diagnose field samples from batches 2 and 3. DNA
160 from all five pools of clinically healthy fry from batch 2 tested negative for by LCHV (**Table**
161 **2**). In batch 3, LCHV was detected from 5 clinically sick fish with viral loads ranging from
162 18.7 to 115.9 copies per 200 ng DNA (Cq 36.84-34.04) and undetectable in 5 clinically
163 healthy fish (**Table 2**).

164 **DISCUSSION**

165 LCHV and SDDV infections reportedly cause similar gross sign of "scale drop" in infected
166 barramundi (de Groof et al., 2015; Chang et al., 2017). Despite the fact that both SDDV and
167 LCHV have been recently discovered, the "scale drop" syndrome has been recognized in
168 Southeast Asia since 1992 (Gibson-Kueh et al., 2012; de Groof et al., 2015). Therefore, it
169 has raised a concern that both of these pathogens may have been long undiagnosed in farmed
170 barramundi due to unavailability of respective diagnostic tools at that time. Nevertheless,
171 currently several molecular detection methods for SDDV are available to support disease
172 investigation. However, following discovery of LCHV as an emerging virus in Singapore in
173 2017 (Chang et al., 2017), there was no continuous research up-to-date. Although several sets
174 of primers were described in the original patent document by Chang et al. (2017), their
175 detection limits and test specificity remain uninvestigated. The validated qPCR method
176 developed in this study might serve as a useful diagnostic tool for rapid screening of the
177 suspected cases as well as active surveillance and early monitoring of the pathogen for the
178 barramundi aquaculture industry.

179 Detection of LCHV in multiple organs of the clinically sick fish suggests that the virus
180 caused systemic infection, similar to that of SDDV (Senapin et al., 2019; Charoenwai et al.,
181 2019; Sriisan et al., 2020). Interestingly, the liver and kidney tissues which are normally used
182 for PCR diagnostics of fish viruses appeared to be unsuitable for LCHV detection while the

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183 fin seemed to be the best targeted tissue due to its highest viral loads, followed by gills,
184 muscle, spleen and brain. There was a limitation of fish numbers in this study, further
185 comparative analysis should be done with larger sample numbers in order to gain a better
186 understanding of virus tissue tropism as well as viral loads in the fish at different stages of
187 infection. Nevertheless, this knowledge might be useful for establishment of cost-effective
188 and non-destructive sampling strategies of fin and/or gills of farmed fish for periodical
189 monitoring of the LCHV.

190 The present study focused primarily on the development and validation of a sensitive qPCR
191 detection method for LCHV. Apart from LCHV, several pathogens have been reported to
192 cause similar clinical signs of "scale drop" including SDDV (Gibson-Kueh et al., 2012; de
193 Groof et al., 2015; Senapin et al., 2019), a pathogenic strain of *Vibrio harveyi,* and
194 *Tenacibaculum maritimum* (Dong et al., 2017a; Gibson-Kueh et al., 2012). Relatively low
195 viral loads present in the clinically sick fish with scale drop disease-like symptoms suggests
196 that LCHV might be an opportunistic pathogen rather than the true causative agent of the
197 diseased fish investigated in this study. However, identification of other pathogens in field
198 samples was not done in this study. We thus recommend that investigation of the at least four
199 aforementioned agents should be considered for the fish showing scale drop symptoms in
200 order to weigh involvement of each pathogen in field outbreaks.

201

202 **Acknowledgements**

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204

205 **Conflict of interest**

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206 The authors declare no conflict of interest.

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258 **Tables and Figures**

259 **Table 1** LCHV loads in 8 different tissues from 5 barramundi samples in batch 1

Sample	Fish clinical status	Cq and LCHV load*/200 ng template)							
		Liver	Kidney	Spleen	Gills	Fin	Brain	Eyes	Muscle
1	"Scale drop"	ND	ND	33.33 [184]	35.91 [34.3]	34.35 [94.7]	34.4 [82.4]	ND	ND
2	"Scale drop"	ND	ND	36.05 [31.3]	32.39 [339]	34.26 [100]	ND	35.07 [59.3]	34.43 [89.9]
3	"Scale drop"	ND	ND	ND	ND	33.65 [149]	37.32 [13.7]	ND	36.46 [24.0]
4	"Scale drop"	36.02 [32]	ND	35.55 [43]	32.92 [240]	31.52 [597]	36.20 [28.4]	34.81 [70.2]	32.12 [404]
5	Healthy	ND	ND	ND	ND	ND	ND	ND	ND

260 *Cq values are the above number while the LCHV loads are shown in [ ]. Grey highlights
261 samples with LCHV load more than 90 copies. ND, not detected

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263 **Table 2** LCHV detection test results of samples from batches 2 and 3

Batch no.	Sample no.	Fish clinical status	Tested tissue	Cq	LCHV loads/200 ng DNA template
2 (fry)	Pool 1	Healthy	whole body	ND	Negative test
	Pool 2	Healthy	whole body	ND	Negative test
	Pool 3	Healthy	whole body	ND	Negative test
	Pool 4	Healthy	whole body	ND	Negative test
	Pool 5	Healthy	whole body	ND	Negative test
3 (juvenile)	Fish no. 1	Healthy	spleen	ND	Negative test
	Fish no. 2	Healthy	spleen	ND	Negative test
	Fish no. 3	Healthy	spleen	ND	Negative test
	Fish no. 4	Healthy	spleen	ND	Negative test
	Fish no. 5	Healthy	spleen	ND	Negative test
	Fish no. 6	"Scale drop"	spleen	35.39	48.2
	Fish no. 7	"Scale drop"	spleen	34.04	115.9
	Fish no. 8	"Scale drop"	spleen	35.48	45.4
	Fish no. 9	"Scale drop"	spleen	36.23	27.9
	Fish no. 10	"Scale drop"	spleen	36.84	18.7

264 ND, not detected

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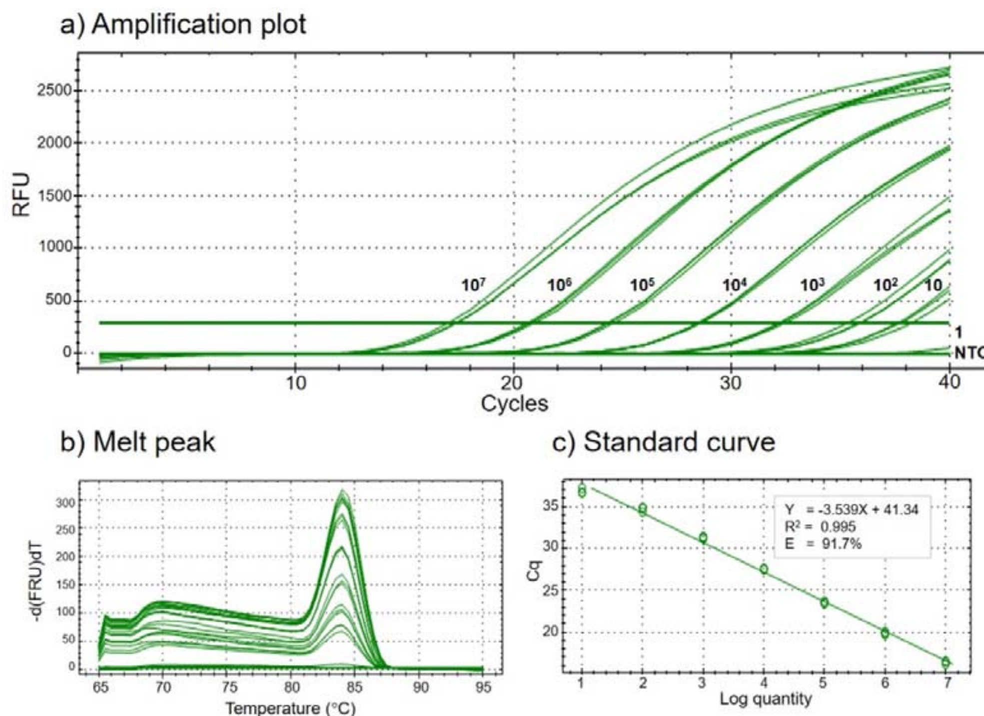

277 **Fig. 1** Performance and sensitivity of LCHV SBYR Green-based qPCR. (a) Amplification
278 plots of positive control plasmid pMEP93 serial dilutions from 10^7 to 1 copies containing 100
279 ng spiked fish DNA in each reaction. Three technical replicates were done for each dilution.
280 (b) Melt peak analysis of the products obtained in (a). (c) Standard curve derived by plotting
281 Cq values versus $\log_{10}$ pMEP93 concentrations. Formula for copy number calculation, R^2 and
282 E values are shown in the box.

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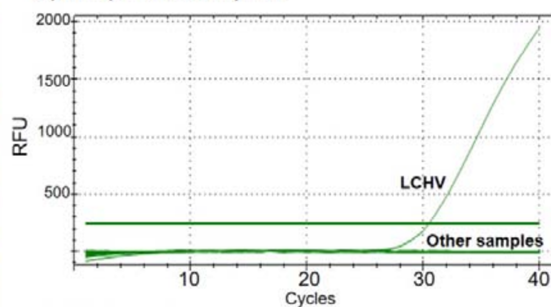
a) List of samples used in specificity test

Pathogens/samples	Code	Source	Ave. Cq
Streptococcus iniae	VN2396	Laboratory strain	ND
Vibrio harveyi	SDMN-Y6	Dong et al. 2017a	ND
Vibrio parahaemolyticus	XN89	Phiwsaiya et al. 2017	ND
Vibrio tubiashi	SDMN-G4	Dong et al. 2017a	ND
Vibrio vulnificus	-	Laboratory strain	ND
Vibrio alginolyticus	-	Laboratory strain	ND
Vibrio cholera	NK8	Dong et al. 2015	ND
Pleisiomas shigelloides	NK10	Dong et al. 2015	ND
Tenacibaculum litopenaei	SDMN-T4	Dong et al. 2017a	ND
Nocardia seriolae	VN2391	Laboratory strain	ND
Aeromonas hydrophila	-	Laboratory strain	ND
Aeromonas dhakensis	-	Laboratory strain	ND
ISKNV infected tissue	-	Dong et al. 2017b	ND
NNV infected tissue	-	Laboratory sample	ND
SDDV infected tissue	-	Senapin et al. 2019	ND
LCHV infected tissue	-	**This study**	**30.35**
Healthy fish	-	Laboratory sample	ND
No template control	-	-	ND

ND, not detected



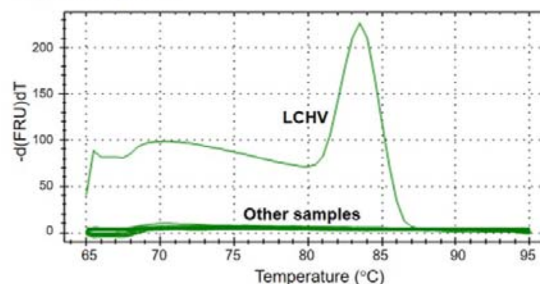


Fig. 2 Specificity test of LCHV SBYR Green-based qPCR. (a) DNA samples extracted from bacterial isolates and viral infected fish as well as control reactions (DNA from healthy fish and no template control) were used in the specificity assay. Average Cq values from technical replicates are shown. ND, not detected. (b) Amplification plots and (c) melt peak analysis of products from samples shown in table (a).

```
gtacttcatcgcctacggagcgctggtcaccctgtacatcataaccaccatgggcctcacg
  Y  F  I  A  Y  G  A  L  V  T  L  Y  I  I  T  T  M  G  L  T
gacgtgaccctggacctcctcaagcacacgta
 D  V  T  L  D  L  L  K  H  T
```

Supplemental Fig. 1 Nucleotide sequence of the LCHV qPCR target. Putative translated amino acid sequence is shown in capital alphabets. qPCR primers (double underlined) were designed to generate a 93-bp fragment of LCHV *MEP* gene. Compared to previously documented sequence (Chang et al. 2017), there is one silent mutation (gray highlighted) found in this study.