Deficiencies in the current assays for the detection and identification of DNA viruses of carp: an assay redesign and evaluation.

David Stone[1], Peng Jia[2] and Hong Liu[2]

[1]Cefas Weymouth Laboratory, UK
[2]Shenzhen Exit & Entry Inspection and Quarantine Bureau, People's Republic of China.

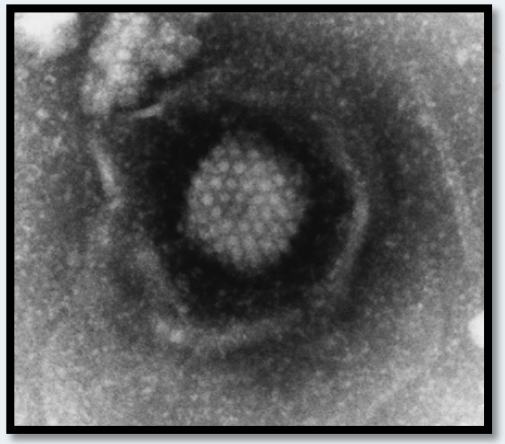
Centre for Environment Fisheries & Aquaculture Science

World Class Science for the Marine and Freshwater Environment

Cefas

Overview

- ~~BREXIT~~
- *Cyprinivirus*-specific primers
- Failures in CyHV-3 detection using the Gilad qPCR assay
- Design and initial evaluation of a CyHV-3 pol qPCR assay
- CEV
- Current PCR based assays
- Failures in the Cefas conventional PCR assay
- Design and initial evaluation of a modified nested PCR assay
- Work to be done



ELSEVIER

Advances in Virus Research

Available online 29 April 2015

In Press, Corrected Proof — Note to users

Cyprinid Herpesvirus 3: An Archetype of Fish Alloherpesviruses

Maxime Boutier*, Maygane Ronsmans*, Krzysztof Rakus*, Joanna Jazowiecka-Rakus*, Catherine Vancsok*, Léa Morvan*, Ma. Michelle D. Peñaranda*, David M. Stone†, Keith Way†, Steven J. van Beurden‡, Andrew J. Davison§, Alain Vanderplasschen*

- Large DNA virus (295 kbp genome) – of the *Alloherpesviridae* family in the order *Herpesvirales*
- CyHV-3 (Koi herpesvirus - KHV) is the type species of the *Cyprinivirus* genus -also contains Cyprinid herpesviruses 1 & 2 and Anguillid herpesvirus
- Disease affects Common carp (*Cyprinus carpio),* including ornamental koi carp and varieties and hybrids such as mirror and ghost carp. Goldfish (*Carassius auratus*) x common carp hybrids also have low susceptibility to CyHV-3 infection

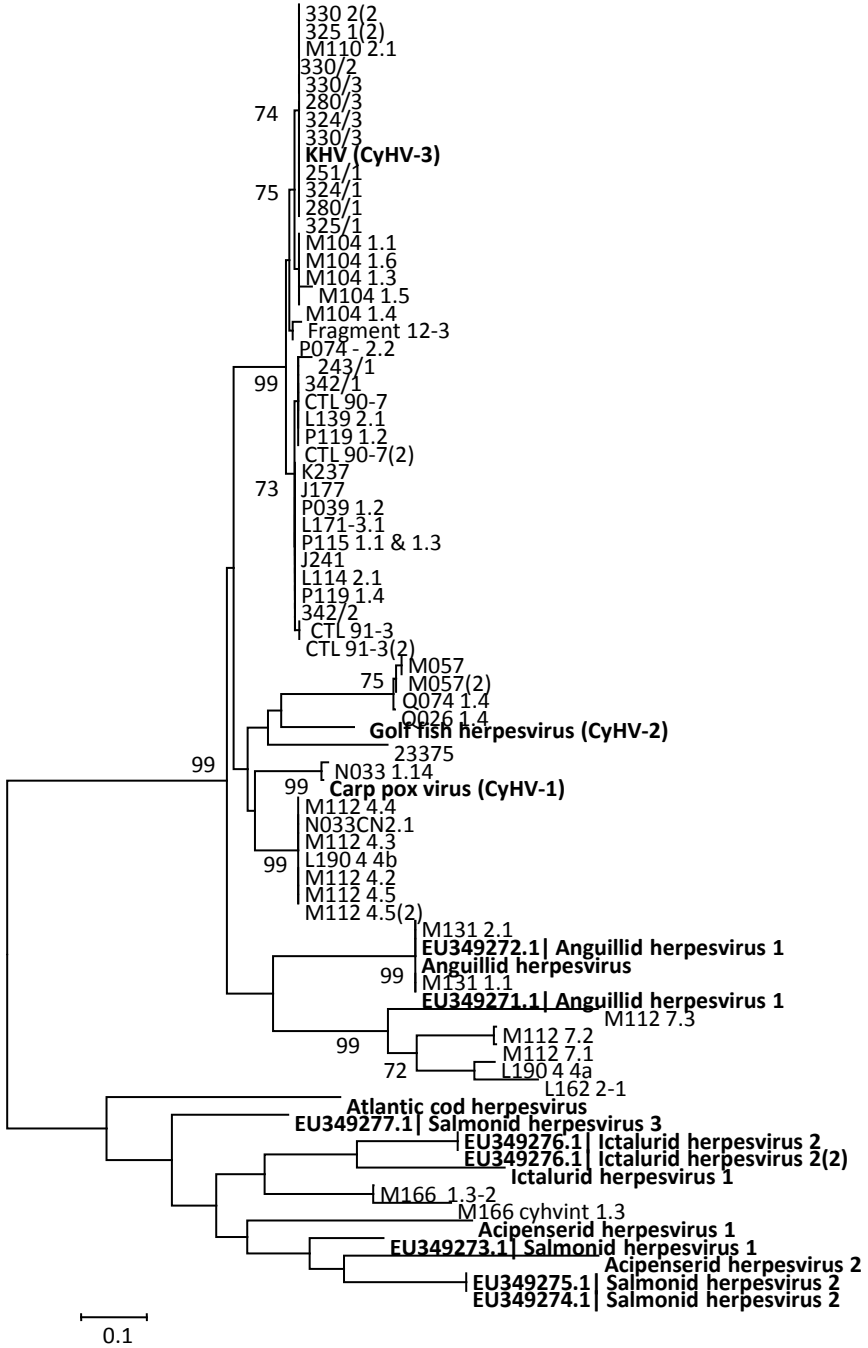
Cyprinivirus- specific DNA polymerase primers

Nested conventional PCR assay based on CyHV 1-3 DNA polymerase sequences

- Analytical sensitivity of 1-10 copies/reaction (~DNA from 0.25mg tissue)
- Assay accredited to ISO 17025

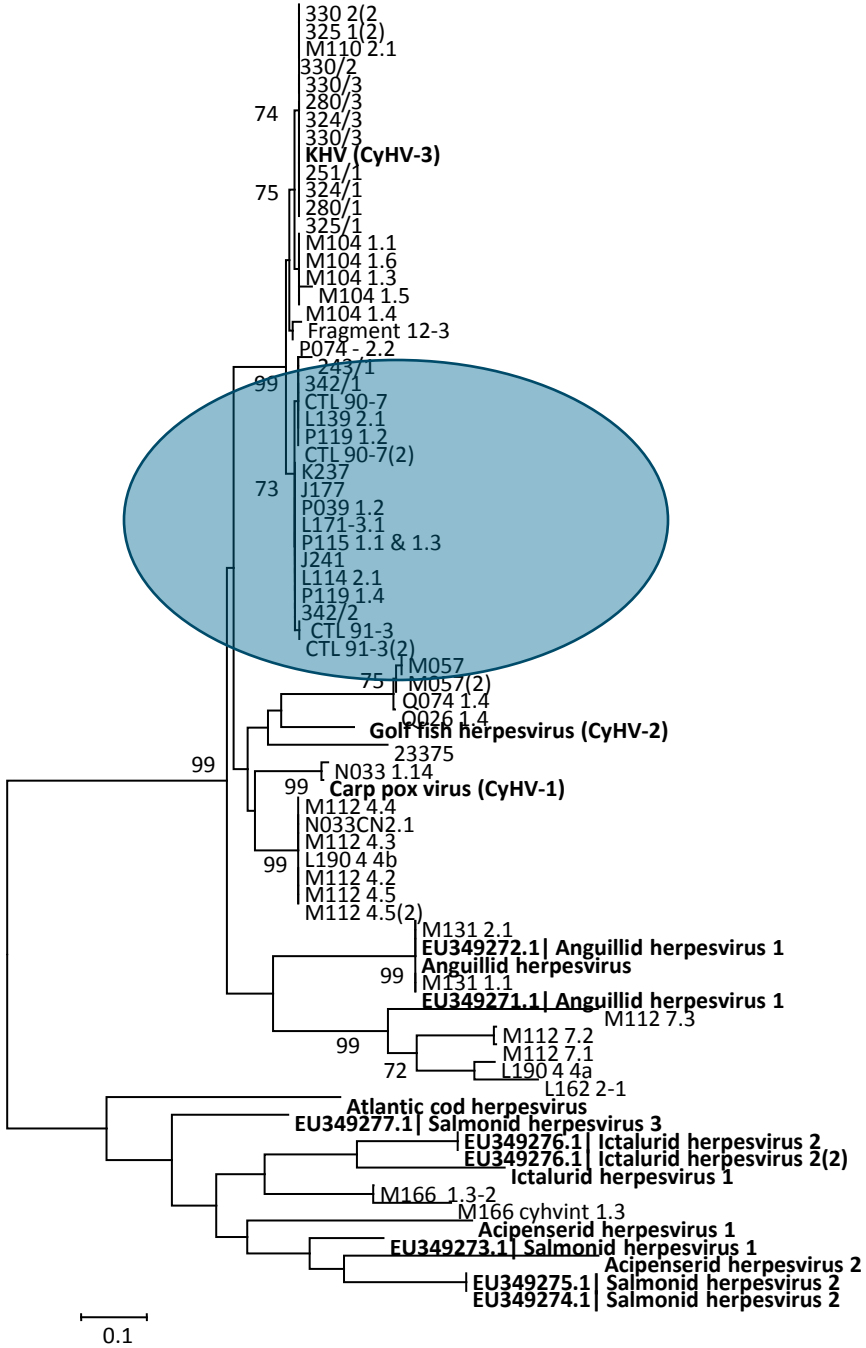
Initially run in parallel to the TK primers recommended by the OIE.

In the UK the assay was adopted as the primary assay for confirmation of disease outbreaks as it performed better in our hands



Herpesvirus diversity

N-J tree based on partial cyprinid herpesvirus DNA polymerase gene sequence (300nt) obtained from carp tissues using the CyHV pol generic primers



CyHV-3 variants

N-J tree based on partial cyprinid herpesvirus DNA polymerase gene sequence obtained from carp tissues using the CyHV pol generic primers

Vol. 107: 113–120, 2013
doi: 10.3354/dao02666
DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org
Published December 12

Detection of novel strains of cyprinid herpesvirus closely related to koi herpesvirus

Marc Y. Engelsma[1], Keith Way[2], Melanie J. Dodge[2], Michal Voorbergen-Laarman[1], Valentina Panzarin[3], Miriam Abbadi[3], Mansour El-Matbouli[4], Helle Frank Skall[5], Søren Kahns[5,6], David M. Stone[2,*]

[1]Central Veterinary Institute (CVI), part of Wageningen UR, PO Box 65, 8200 AB Lelystad, The Netherlands
[2]Cefas, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK
[3]Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Viale dell'Universita 10, 35020 Legnaro, Padova, Italy
[4]University of Veterinary Medicine, Vienna, Veterinärplatz 1, 1210 Vienna, Austria
[5]National Veterinary Institute (DTU-Vet), Technical University of Denmark, Hangovej 2, 8200 Aarhus N, Denmark
[6]*Present address:* Danish Technological Institute, Kongsvang Allé 29, 8000 Aarhus C, Denmark

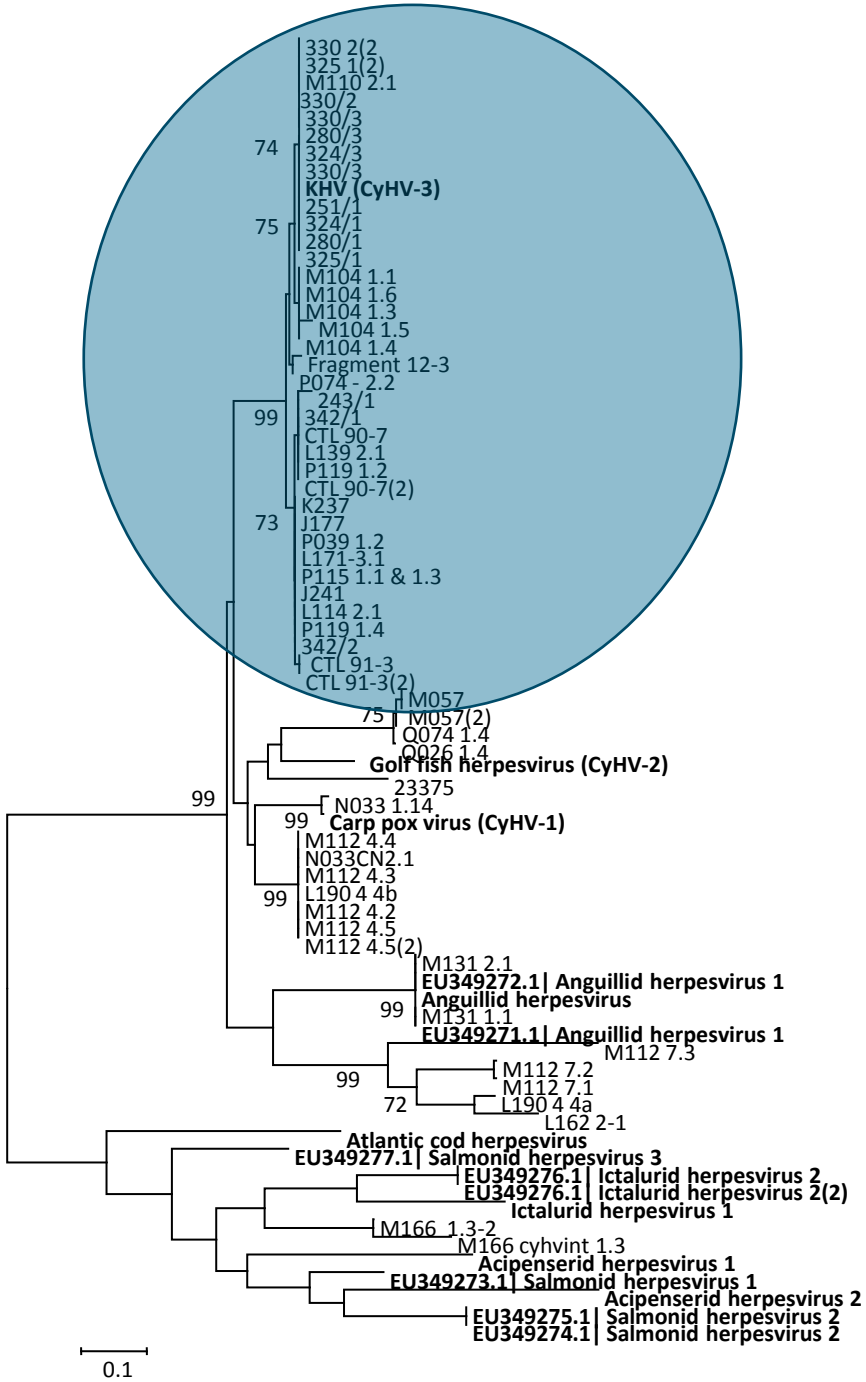
Significance of the findings

- The significance of the novel CyHV-3 strains for common carp is not fully understood
- Many of the CyHV-3 variants were detected in apparently healthy animals.

  low-pathogenic strains of CyHV-3 that do not warrant control in the same way as conventional CyHV-3 isolates.

- In a few cases the animals exhibited signs usually attributed to a CyHV-3 infection

  pathogenic strains of CyHV-3 that do warrant control.

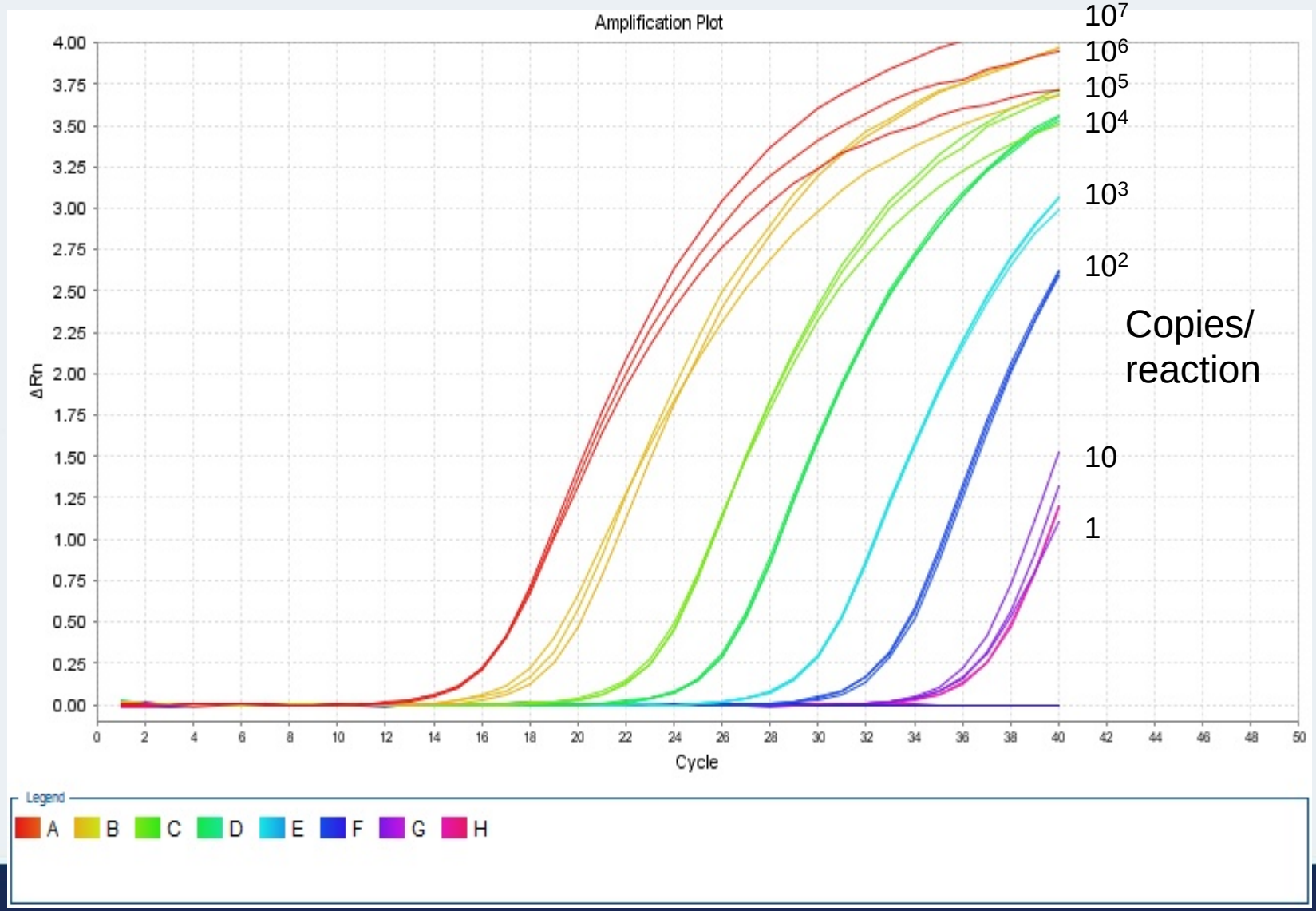


KHV

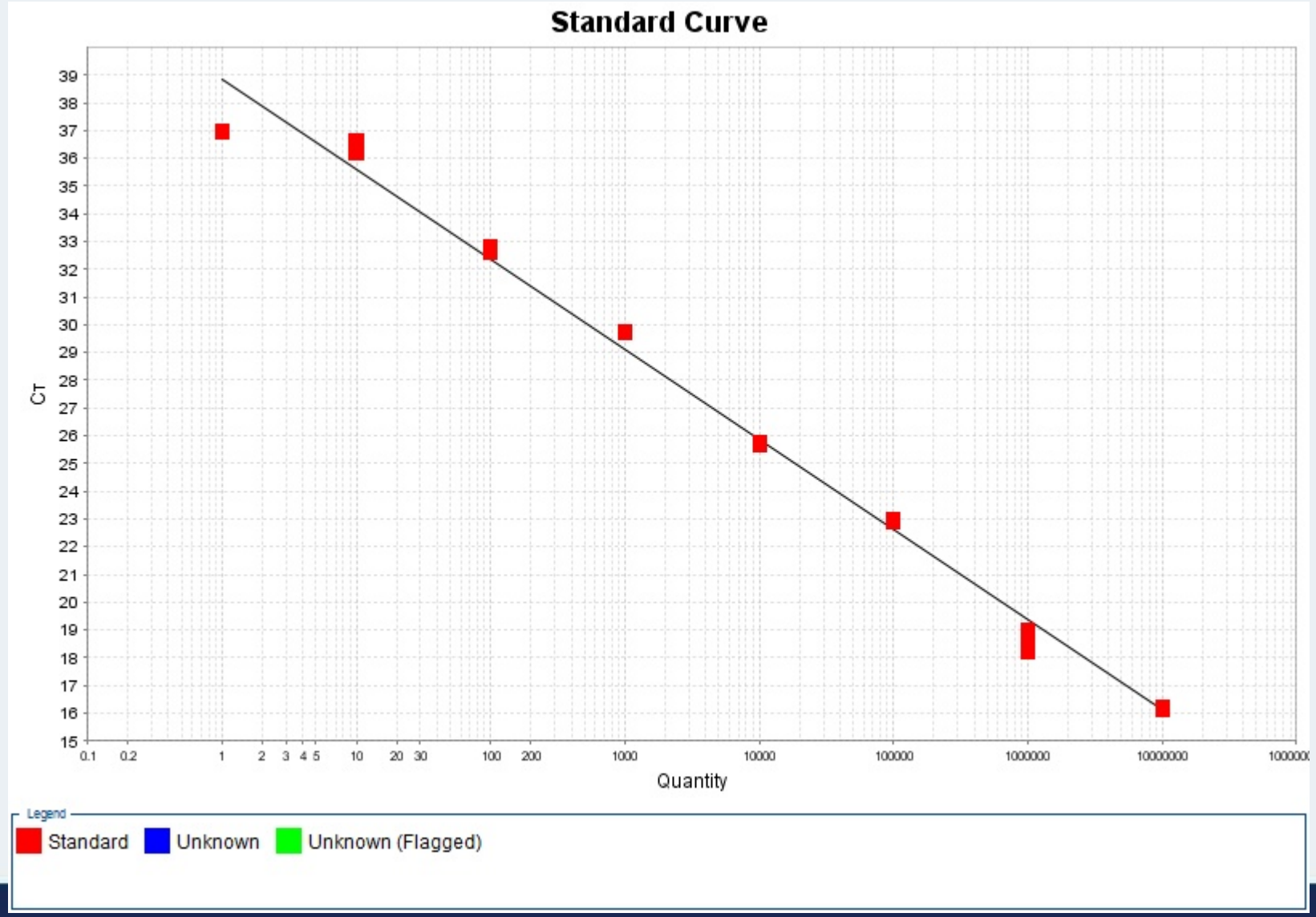
All strains of CyHV-3 should to be treated as having the potential to cause disease.

CyHV-3 qPCR assay design

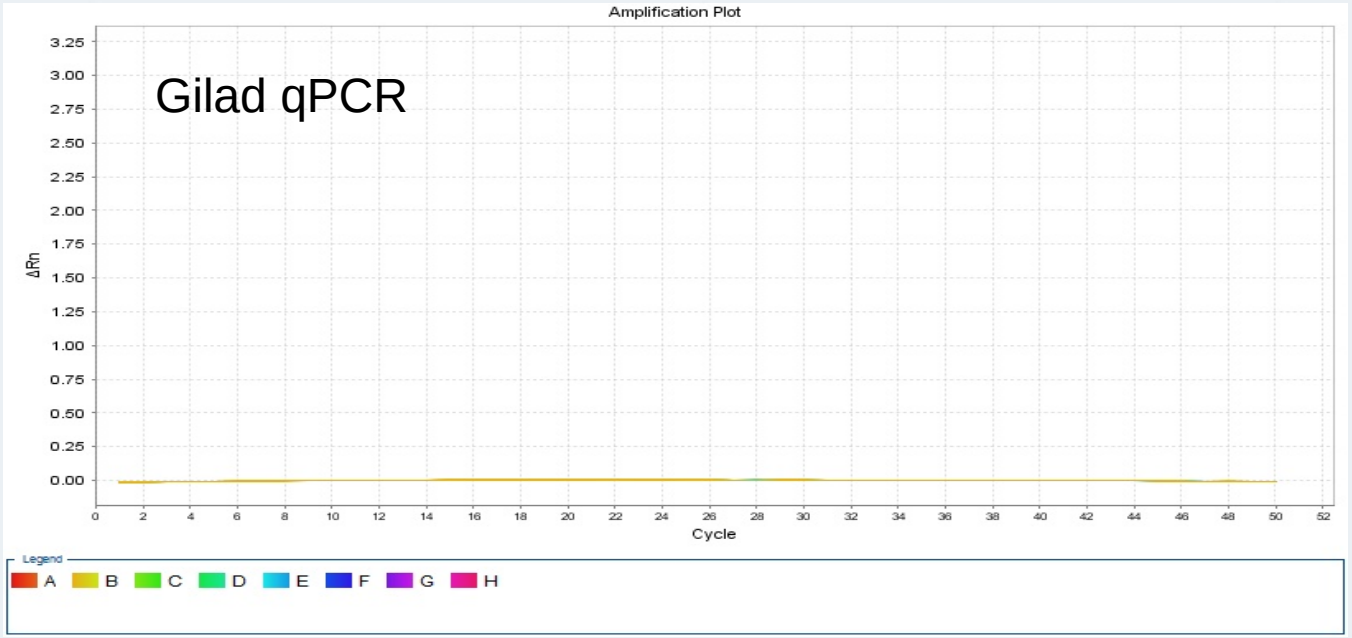
- Multiple alignment of CyHV-1-3 partial DNA polymerase sequences using Clustal W
- Design multiple primer and probe combinations
- Select the assay that performed well

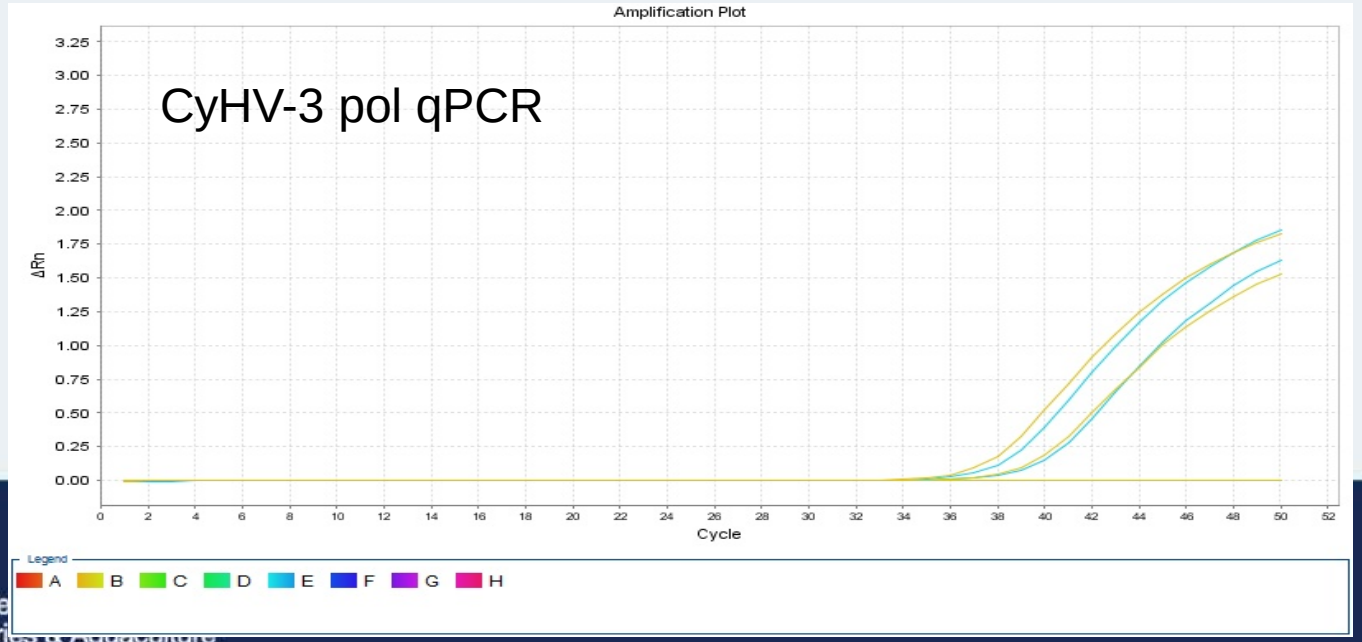


CyHV-3 qPCR amplification curves using serially diluted plasmid and carp DNA extracts as a matrix

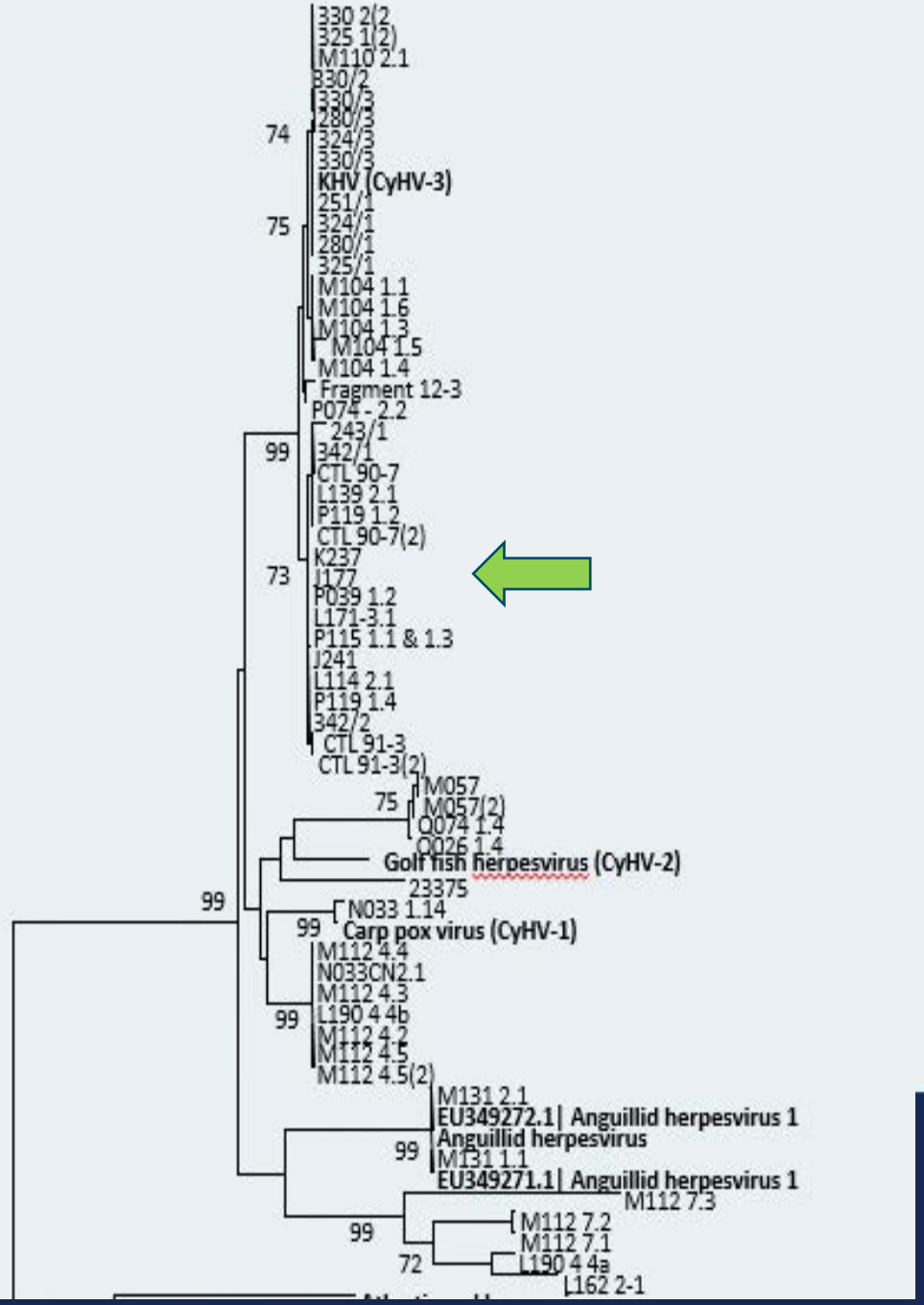


CyHV-3 qPCR standard curve

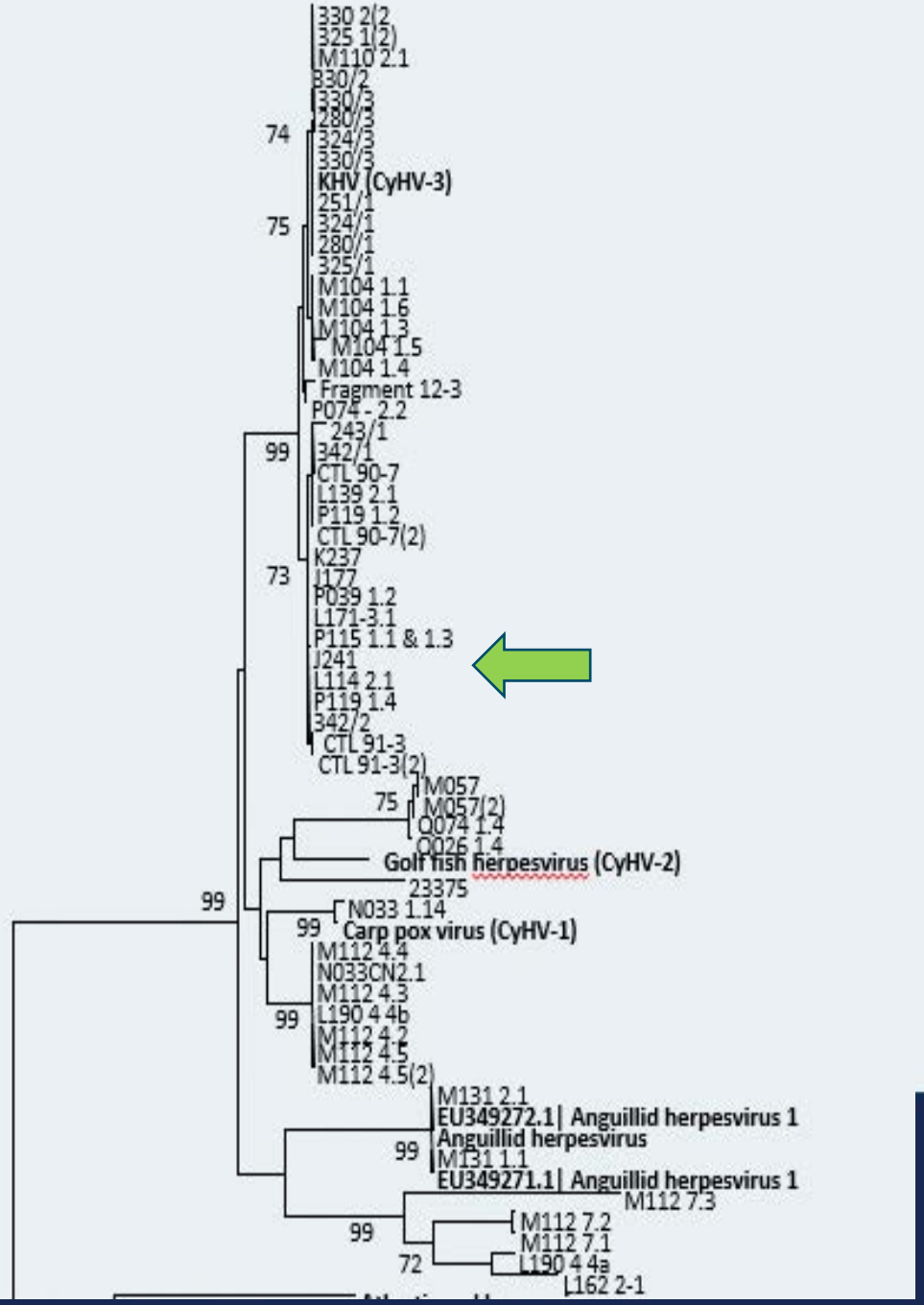




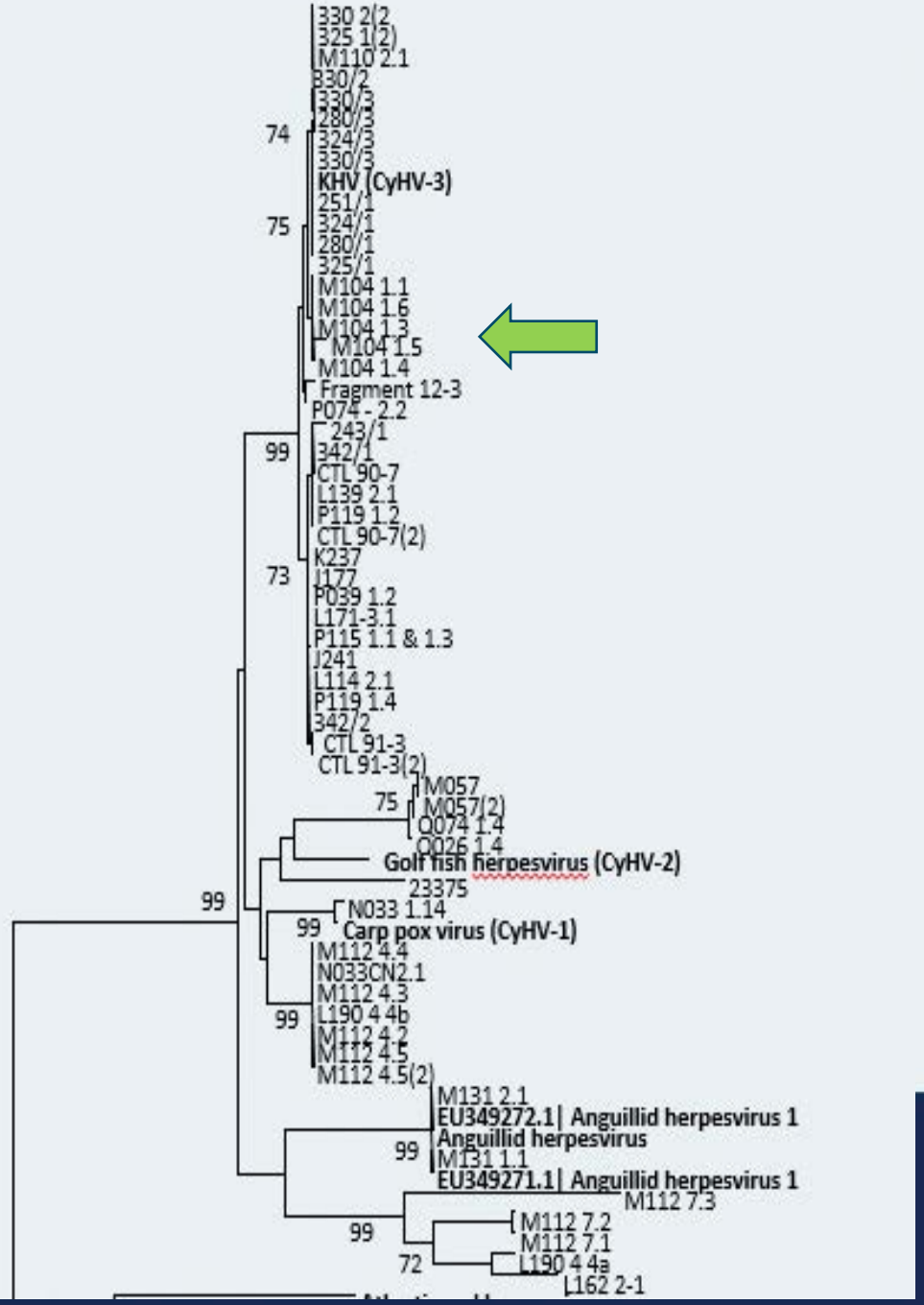
Gilad qPCR and CyHV-3 pol qPCR amplification curves using DNA extracted from tissue infected with a CyHV-3 variants



Sample	Gilad et al qPCR	TK conventional PCR	KHV pol qPCR	CyHV pol conventional PCR
J177 4.1	-ve	-ve	+ve	+ve
J241 2.1	-ve	-ve	+ve	+ve
L191 1.3	-ve	-ve	+ve	+ve
M104 1.3	-ve	-ve	+ve	+ve
L147 2.1 CyHV-1	-ve	-ve	-ve	+ve
N092 1.2 CyHV-1	-ve	-ve	-ve	+ve
L119 4.1 CyHV-1	-ve	-ve	-ve	+ve
M119 2.1 CyHV-3	+ve	+ve	+ve	+ve
M112 4.3	-ve	-ve	-ve	+ve

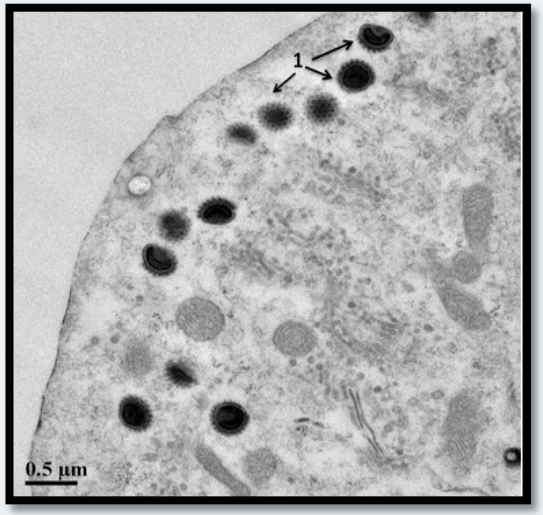


Sample	Gilad et al qPCR	TK conventional PCR	KHV pol qPCR	CyHV pol conventional PCR
J177 4.1	-ve	-ve	+ve	+ve
J241 2.1	-ve	-ve	+ve	+ve
L191 1.3	-ve	-ve	+ve	+ve
M104 1.3	-ve	-ve	+ve	+ve
L147 2.1 CyHV-1	-ve	-ve	-ve	+ve
N092 1.2 CyHV-1	-ve	-ve	-ve	+ve
L119 4.1 CyHV-1	-ve	-ve	-ve	+ve
M119 2.1 CyHV-3	+ve	+ve	+ve	+ve
M112 4.3	-ve	-ve	-ve	+ve



Sample	Gilad et al qPCR	TK conventional PCR	KHV pol qPCR	CyHV pol conventional PCR
J177 4.1	-ve	-ve	+ve	+ve
J241 2.1	-ve	-ve	+ve	+ve
L191 1.3	-ve	-ve	+ve	+ve
M104 1.3	-ve	-ve	+ve	+ve
L147 2.1 CyHV-1	-ve	-ve	-ve	+ve
N092 1.2 CyHV-1	-ve	-ve	-ve	+ve
L119 4.1 CyHV-1	-ve	-ve	-ve	+ve
M119 2.1 CyHV-3	+ve	+ve	+ve	+ve
M112 4.3	-ve	-ve	-ve	+ve

Conclusions for the CyHV-3 assays



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https://doi.org/10.3354/dao03164

DISEASES OF AQUATIC ORGANISMS
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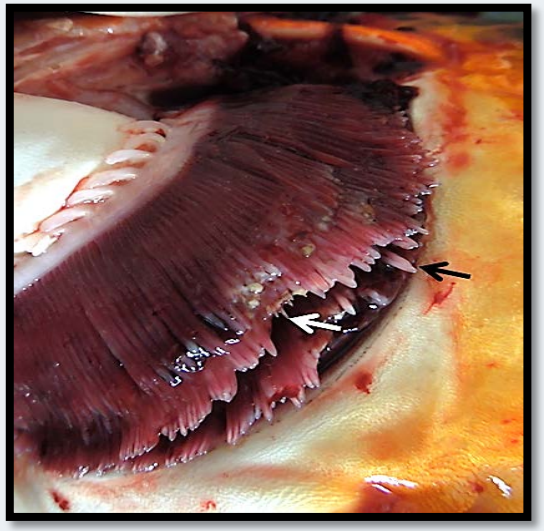
Published October 18

REVIEW

Emergence of carp edema virus (CEV) and its significance to European common carp and koi *Cyprinus carpio*

K. Way[1,*], O. Haenen[2], D. Stone[1], M. Adamek[3], S. M. Bergmann[4], L. Bigarré[5], N. Diserens[6], M. El-Matbouli[7], M. C. Gjessing[8], V. Jung-Schroers[3], E. Leguay[9], M. Matras[10], N. J. Olesen[11], V. Panzarin[12], V. Piačková[13], A. Toffan[12], N. Vendramin[11], T. Veselý[14], T. Waltzek[15]

[1]Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, DT4 8UB, UK
[2]Wageningen Bioveterinary Research of Wageningen UR, 8219 PH, Lelystad, The Netherlands
[3]Fish Disease Research Unit, University of Veterinary Medicine, 30559 Hannover, Germany
[4]Friedrich-Loeffler-Institut, 17493 Greifswald, Insel Riems, Germany
[5]ANSES, Fish Viral Pathology, 29280 Plouzané, France
[6]Centre for Fish and Wildlife Health, University of Bern, 3012 Bern, Switzerland
[7]Clinical Division of Fish Medicine, University of Veterinary Medicine, 1210 Vienna, Austria
[8]Norwegian Veterinary Institute, 0106 Oslo, Norway



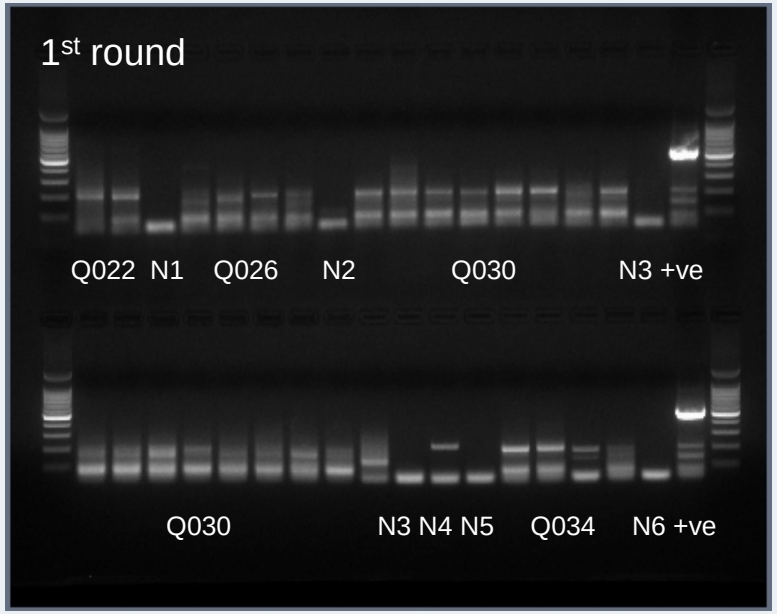
Large DNA virus. Genome ~ 460 kbp (Thomas Waltzek (personal communication)

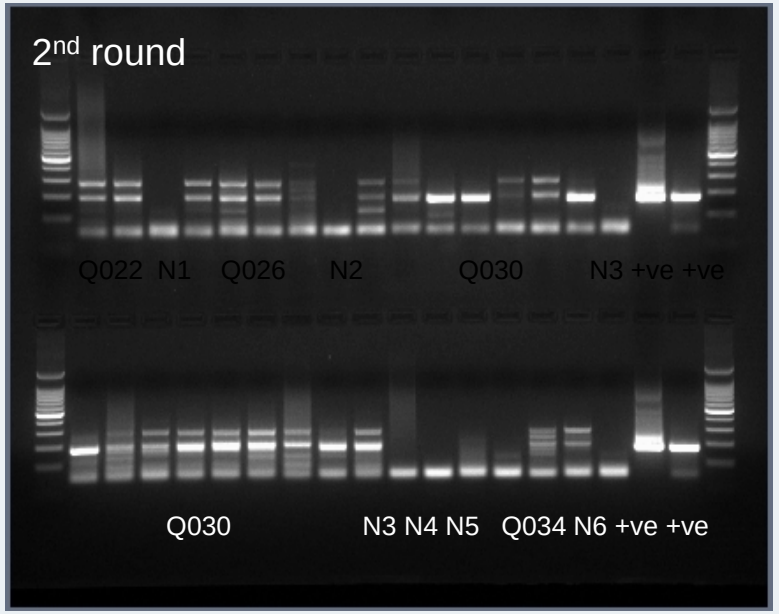
The disease has been reported to generally occur naturally in koi at water temperatures between 15-25°C with a cumulative mortality that may reach 75-100% in juvenile koi, but outbreaks have also been reported to occur during periods of low water temperatures (6-10°C)

Design of a new assay prompted by CEV outbreak in common carp 2012

- Carp mortality in several ponds in London to the South of the river Thames.
- Initial confirmation of CEV in these cases was achieved by PCR using the primers described by Oyamatsu et al. (1997)
- No products in the 1st round PCR. Non-specific products of a similar size to the expected product in the 2nd round which made the interpretation of the assay difficult

Non-specific PCR results using the using the primers described by Oyamatsu et al. (1997)

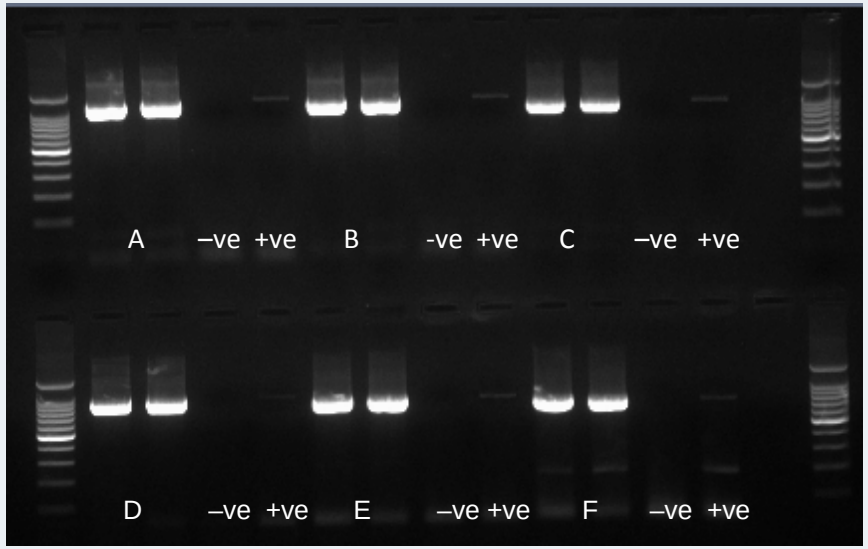


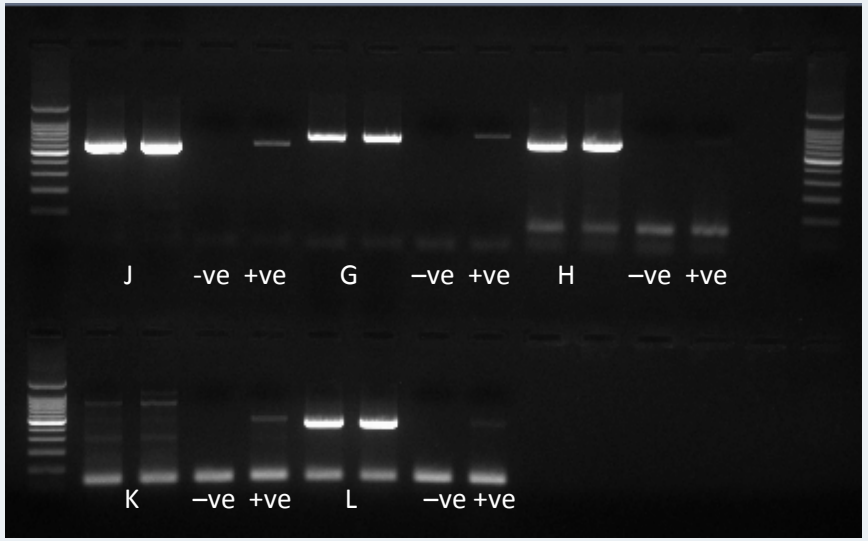


Design of PCR primers based on the based on the CEV sequence obtained from T Miyazaki

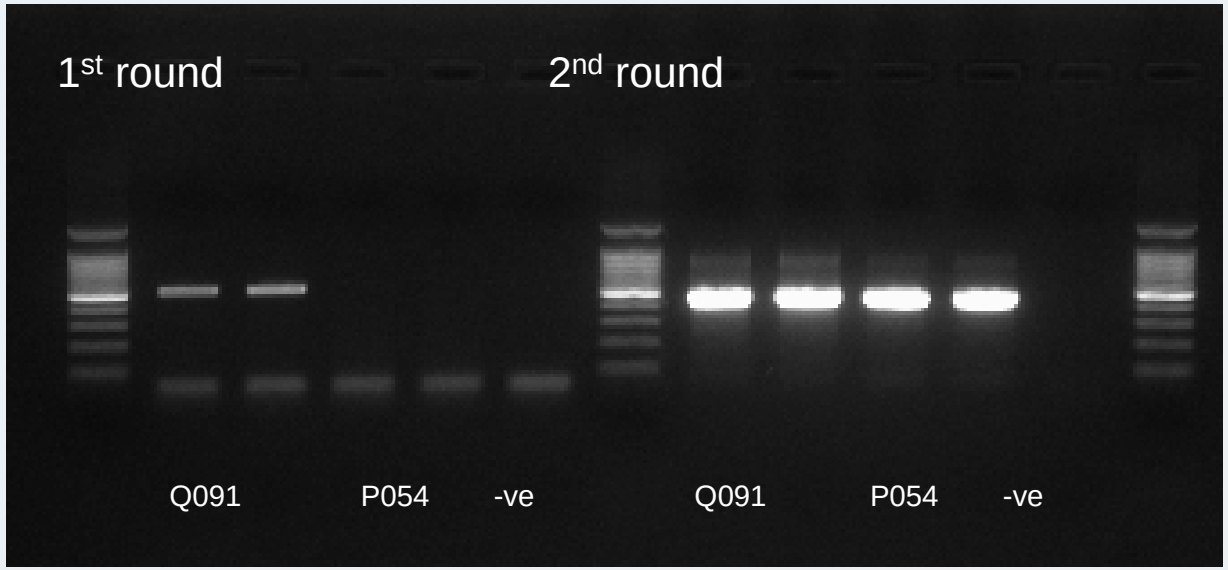
```
>CyPP-3
       CEV ForA                                                    CEV ForB
TGCAGGATCATTATCCTTGTATCTACAAGTACAATAGAAAGCAAGAAGTTTGTATGGAGTATCCAAAGTACTTAGATTA

ATGTTATCAATGAAATTTGTGTATTGTGTTTTTGTTAGTCCAAGAGTTTTCTTCTCATCGTTTGTTACCTTTTGTAGTT
            CEV ForC
GTTTAATATTTGTGATAAGATTTCCATTAGCATAAAATCCTTCCCAAATTTGTGTTGATACATGTTTTAGTGTTTTGTA
            CEV ForD                                                CEV ForE
GATTGTAGCATTTCCTAGTTTGTATGGCAAGAAACAAACTCTCTTTACTGCAACTCCTTGAGGAATTTGATCTAGAATT
                                                                    CEV ForF
CCACAGAATGTAATCTCAAATTTGTTTGTAGAGTTTTTGAAGTATACTGTTTCATCACACAATCCTAGAACTAGAGCAA
                        CEV ForG
GATTAGAAGTCATTGTCTTATCGAAGACATTCATCTTATTCCAATCATCAATCTGAATTCCTTTCCAGAACATAACATT
                        CEV ForH
TGCAATTTTAACTTGCTCTGGAATTGTATCAACATGTCCAATATCTTTCTTTACTACGTAATTTGGATGAGGTAGTACT
                        CEV ForJ
TTGCTAACAAAGTCACAATAGTGAAGAGTTGTCATTTTAATTTGTTGTAGTCCAATTTCTGCAAATTGATATATATCAG
                        CEV ForK                                    CEV ForL
GAATATCAAATTTAACCATATTTGCAAATGGATTTGCTGCTGGTGCTGCCATTACGTAATTAGAATCGCGAAGTTCAGG
                  CEV For1
ATCTCTTGCTGCTGCTGTTGCAACCATTTGAGAATGAACCGAATCAACAAGTTGATATGCTTTTGCATTTGCATCAAAA
GCAACAACTTGACGAGGGAATGATTGGGACAAAGTAGAACTTCTTGTATAATGTATATCTTGAGAAGCAGCTGCTCCAC
CTGCTACAATTCCAAGAGCATAATGATATTCAAGATCTAGTTTAATTTGATCTGGAGAATAAGTGTATGCCTTAATTCC
ATATAGCTTAATGAAATGCTCATAATTACCTTGTCCAAACAAAGTTAGGTAAAATAGTTTTAGGATTGAAGCAAGAGCT
GCTGCACTTTTAGGAGGACAAGTAAAGTTACCACCAGCTCCCTACAAGGAAAGCAATTGATTTTATACTTGAAGAACAA
TCTAGAAGATTGGAGAAATTCTCAAGAATTAGAATTGCAACTTCTAGTCTCTCTAGTTTTTCTAGATTTAGATTTAGGT
TGGCATCGAAATAACTTGCATAATCTAGAAGTTCATCAACATCAAATGTACTTACATCAAATAGGAAAGGATTAGGAGC
AACCTGCA
```

Initial PCR results using the primers based on the CEV sequence obtained from T Miyazaki combined with R3 primer from Oyamatsu et al. (1997).

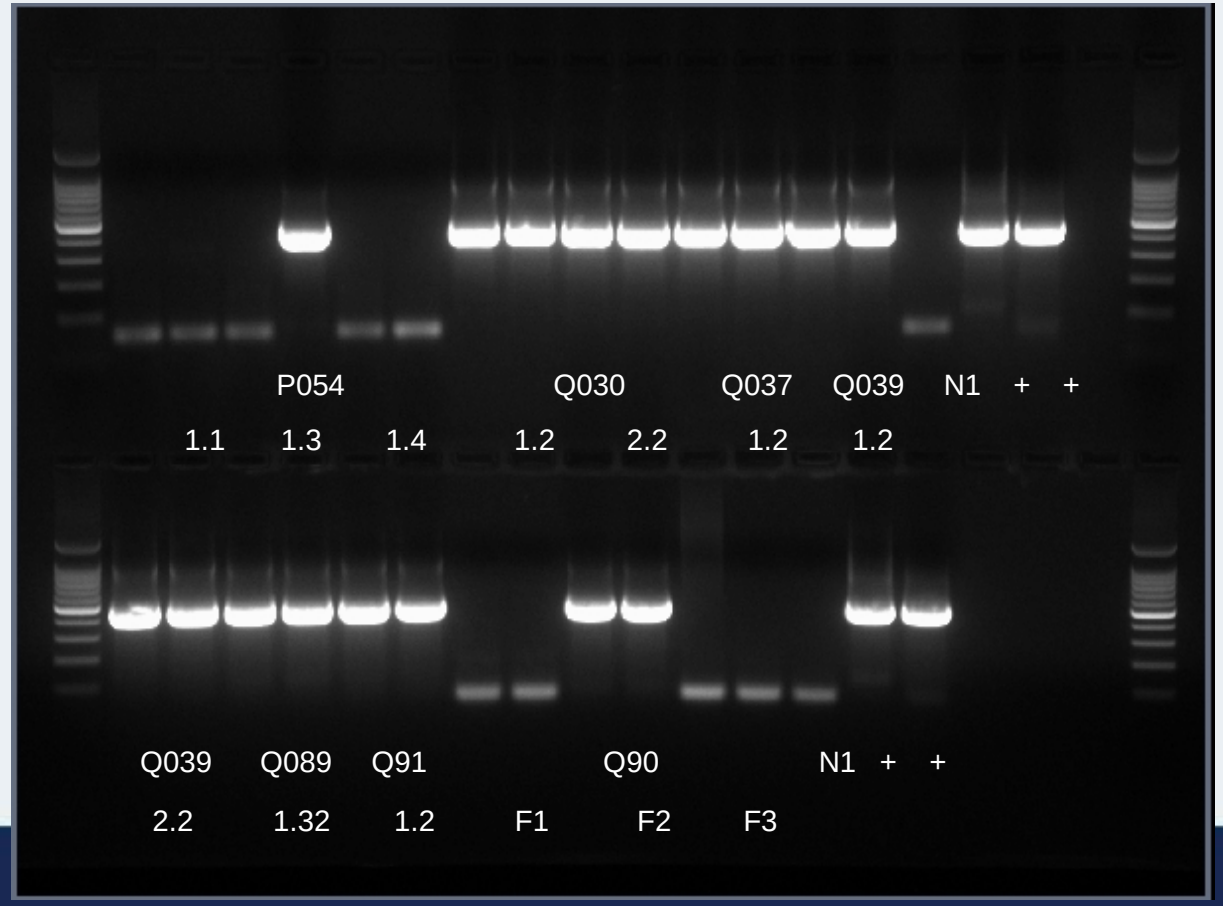




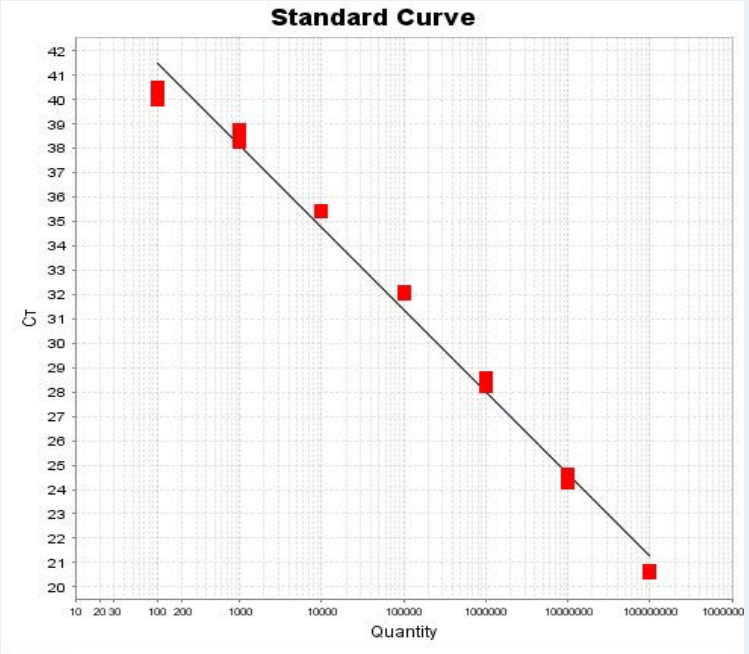
Detection of CEV in gills by PCR using the B/J and B int/J int primer sets

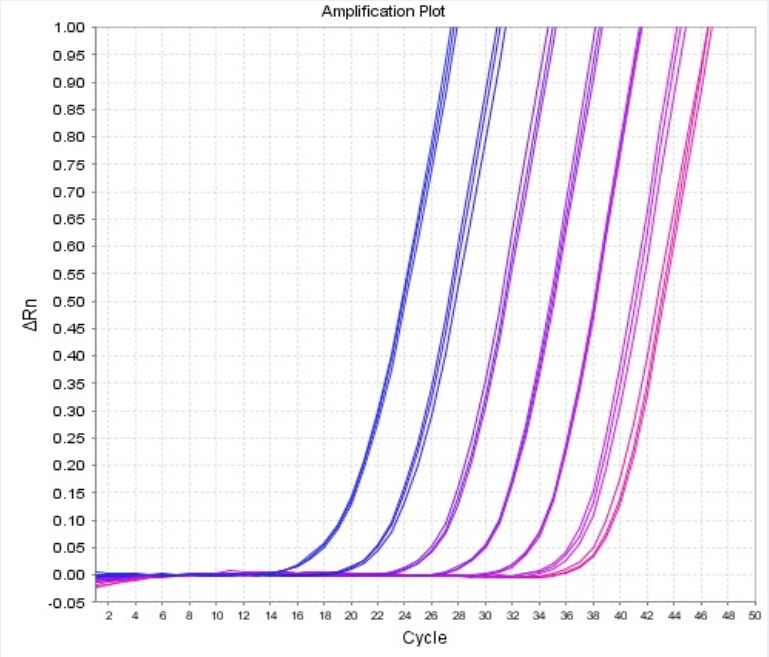


Detection of CEV-like sequences in carp tissue by nested PCR using CEV B/CEV J in the first round and CEV Bint/CEV Jint in the second round.



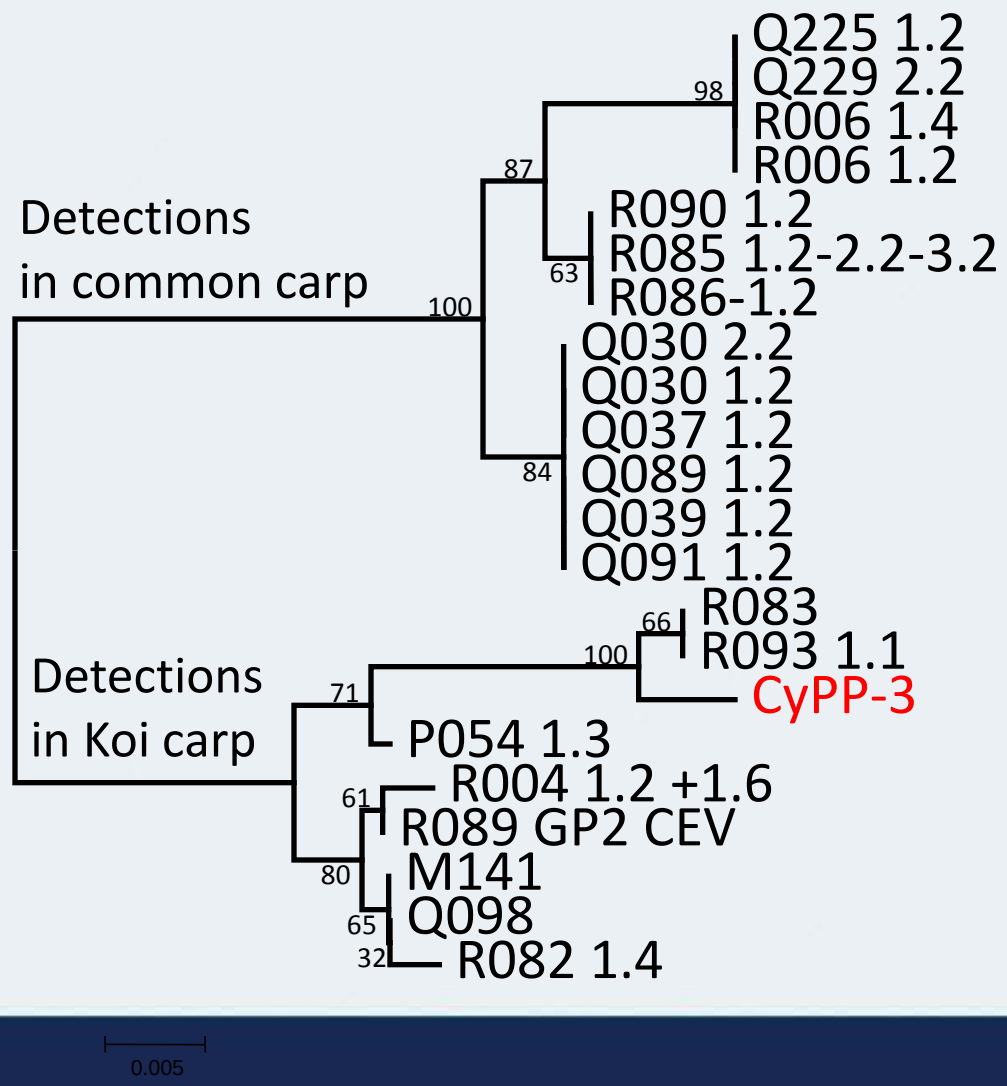
Taqman qPCR assay for CEV





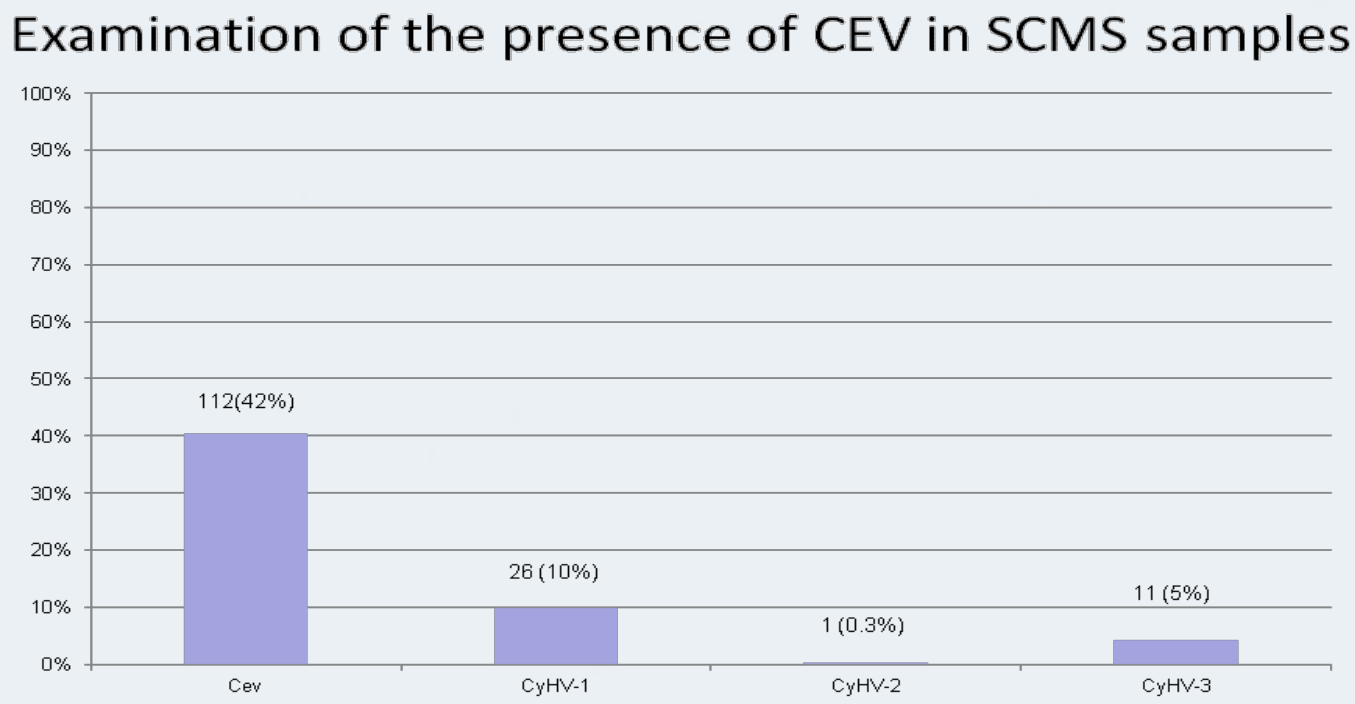
Detection limit of 1-10 copies/reaction

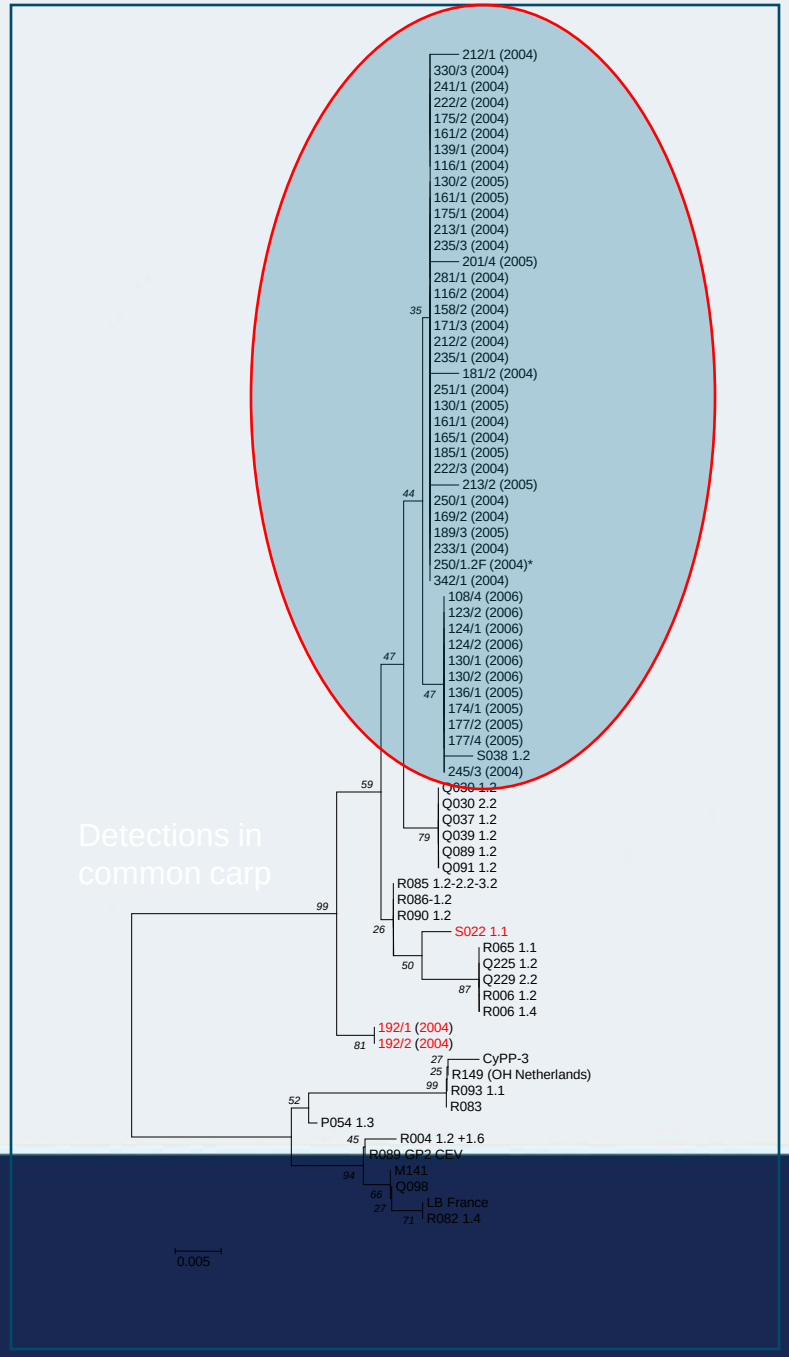
Widely used as a primary screening tool for surveillance and disease confirm by conventional PCR and sequence analysis using the Cefas CEV B/J and Bint/Jint primer sets.



Neighbour-joining tree based on partial sequence (433nt) of the core protein P4a gene of CEV from 2009 – 2013

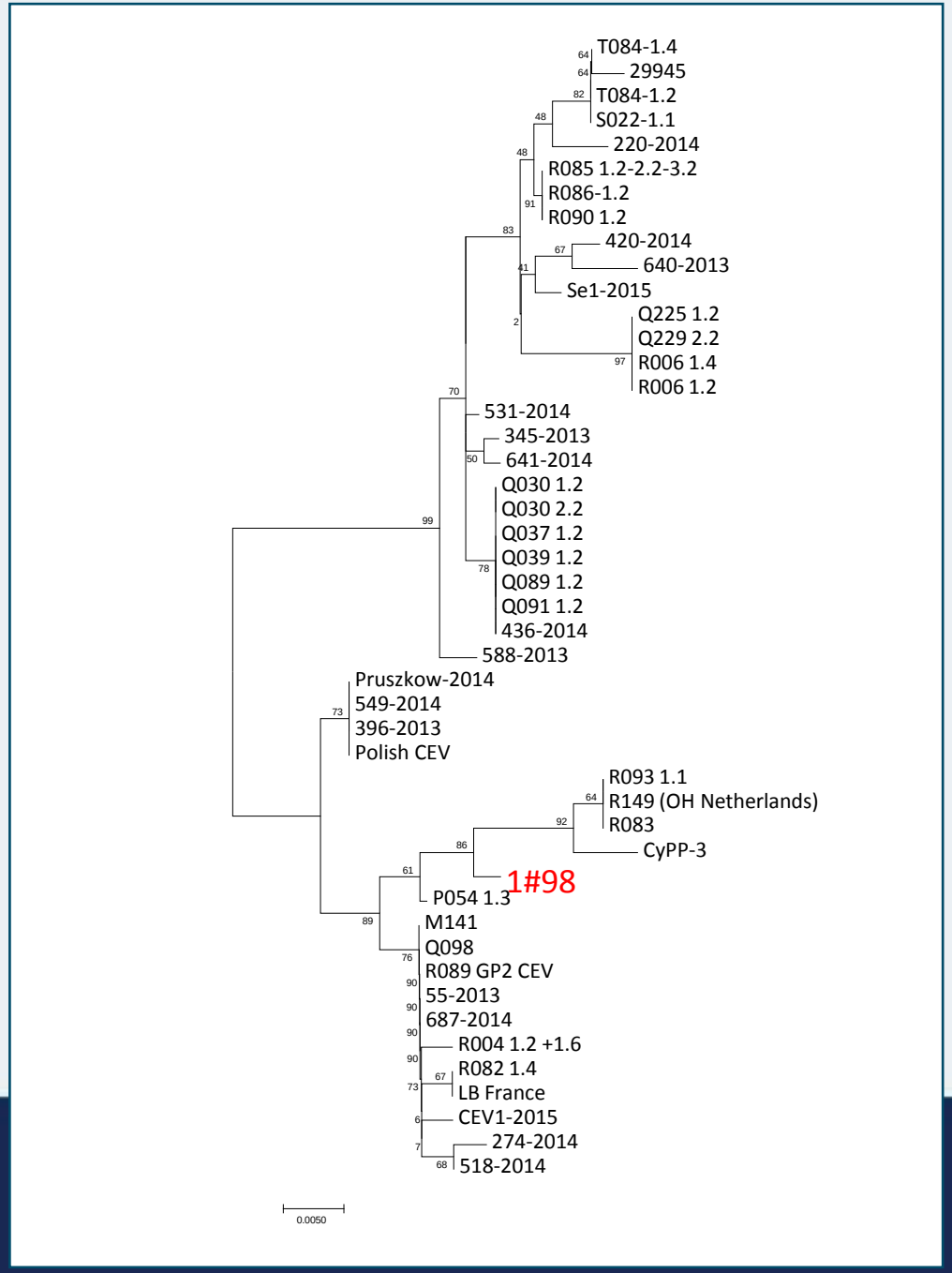
Could this disease be a major contributor to **Spring Carp Mortality Syndrome (SCMS)** – unexplained mortalities, reported to occur in carp fisheries since 1980s ?





All of the CEV-like sequences generated from SCMS tissues samples from 2004-2006 were assigned to the lineage linked common carp.

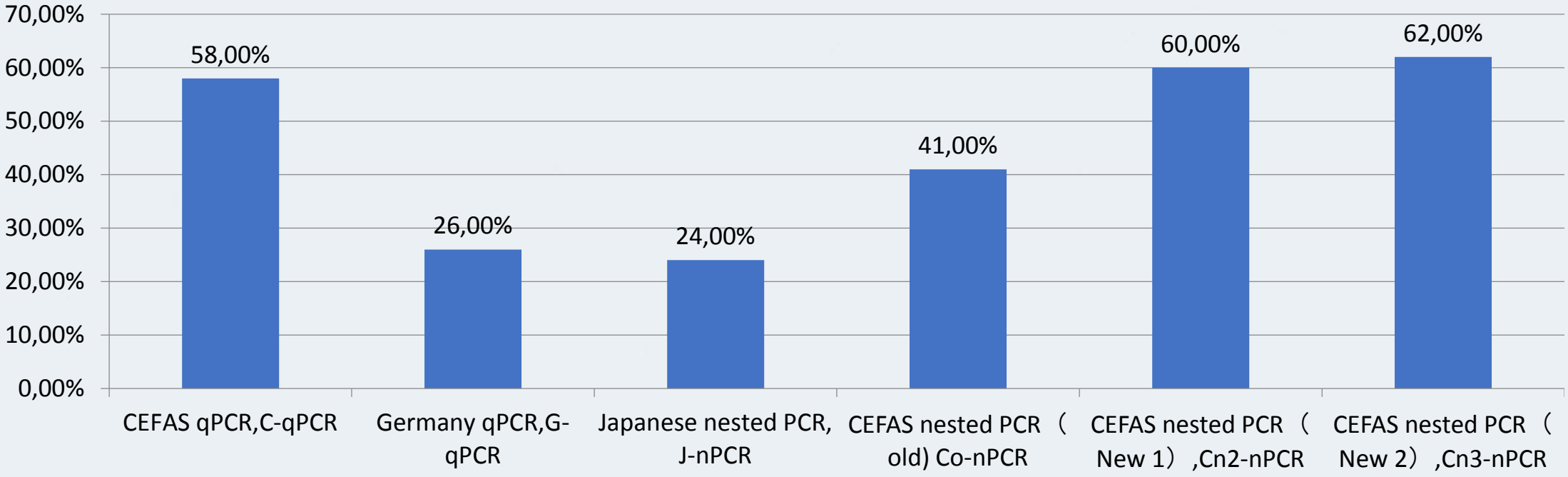
- CEV specific real-time qPCR and conventional PCR
- Adopted internationally as the ‘standard’ for CEV surveillance and disease confirmation.


T084-1.4
29945
T084-1.2
S022-1.1
220-2014
R085 1.2-2.2-3.2
R086-1.2
R090 1.2
420-2014
640-2013
Se1-2015
Q225 1.2
Q229 2.2
R006 1.4
R006 1.2
531-2014
345-2013
641-2014
Q030 1.2
Q030 2.2
Q037 1.2
Q039 1.2
Q089 1.2
Q091 1.2
436-2014
588-2013
Pruszkow-2014
549-2014
396-2013
Polish CEV
R093 1.1
R149 (OH Netherlands)
R083
CyPP-3
1#98
P054 1.3
M141
Q098
R089 GP2 CEV
55-2013
687-2014
R004 1.2 +1.6
R082 1.4
LB France
CEV1-2015
274-2014
518-2014
0.0050

Highly variable IGR sequence

Table 2 the results of nested PCR base on old primers and new primers

Samples	Old primers		New primers 1		New primers 2	
	1st PCR (CEV B/J)	2nd PCR (CEV Bint/Jint)	1st PCR (CEV B/J2)	2nd PCR (CEV Bint/Jint2)	1st PCR (CEV B/J2)	2nd PCR (CEV Bint/Jint3)
ZM8	-	-	+	+	+	+
3#	-	-	+	+	+	+
5#	-	-	-	-	-	-
3979	-	-	-	-	-	-
3800	-	-	-	+	-	+
3801	-	-	-	+	-	+


70,00%
60,00%
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
58,00%
26,00%
24,00%
41,00%
60,00%
62,00%
CEFAS qPCR,C-qPCR
Germany qPCR,G-qPCR
Japanese nested PCR, J-nPCR
CEFAS nested PCR（ old) Co-nPCR
CEFAS nested PCR（ New 1）,Cn2-nPCR
CEFAS nested PCR（ New 2）,Cn3-nPCR

So what have we missed?

Thank You

Exploiting the increased discriminatory power of Variable Number Tandem Repeats (VNTRs)

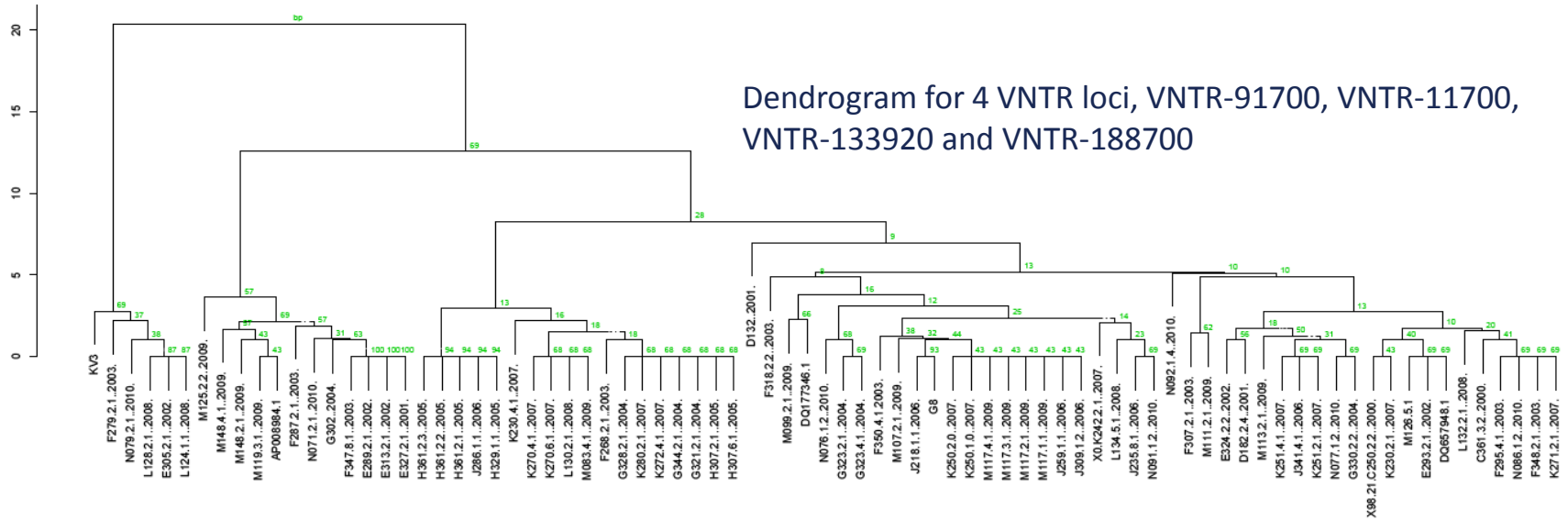
The three complete CyHV-3 genomes aligned using Clustal W and the uninterrupted VNTRs were identified by visual examination

- 15 uninterrupted VNTRs displaying at least 4 repetitions.
- 11 with a repeat unit of 3 or more in length.
- 6 of the 11 alleles differed in repeat number between the CyHV-3 U and CyHV-3 I strains.

VNTR polymorphisms in the CyHV-3 genome

KHV virus strain	Repeat size (nucleotides)	Start position	Repeat sequence
DQ657948.1 DQ177346.1 AP008984.1	3	4810 4798 4752	$(TGA)_4$ $(TGA)_5$ $(TGA)_4$
DQ657948.1 DQ177346.1 AP008984.1	9	11628 11619 11545	$(AGTGAGCAG)_5$ $(AGTGAGCAG)_7$ $(AGTGAGCAG)_4$
DQ657948.1 DQ177346.1 AP008984.1	3	15501 15497 15455	$(TCA)_5$ $(TCA)_5$ $(TCA)_8$
DQ657948.1 DQ177346.1 AP008984.1	2	16100 16096 16052	$(TG)_{46}$ $(TG)_{56}$ $(TG)_{34}$
DQ657948.1 DQ177346.1 AP008984.1	2	37878 37928 37856	$(GT)_{37}$ $(GT)_{22}$ $(GT)_{39}$
DQ657948.1 DQ177346.1 AP008984.1	12	90974 91007 91021	$(AACCACCGAGGA/T)_{18}$ $(AACCACCGAGGA/T)_{18}$ $(AACCACCGAGGA/T)_{37}$
DQ657948.1 DQ177346.1 AP008984.1	6	133275 133307 133358	$(ACCCTC)_7$ $(ACCCTC)_8$ $(ACCCTC)_5$
DQ657948.1 DQ177346.1 AP008984.1	2	177568 177605 177510	$(GA)_{81}$ $(GA)_{47}$ $(GA)_{40}$
DQ657948.1 DQ177346.1 AP008984.1	3	188012 187984 187881	$(TTC)_{23}$ $(TTC)_{28}$ $(TTC)_{14}$
DQ657948.1 DQ177346.1 AP008984.1	9	216450 216418 216286	$(AGCAACAGC)_9$ $(AGCAACAGC)_8$ $(AGCAACAGC)_{10}$
DQ657948.1 DQ177346.1 AP008984.1	9	216553 216513 216398	$(CTTCAGCAC)_4$ $(CTTCAGCAC)_4$ $(CTTCAGCAC)_{18}$
DQ657948.1 DQ177346.1 AP008984.1	3	277488 277451 277589	$(TGA)_4$ $(TGA)_5$ $(TGA)_4$
DQ657948.1 DQ177346.1 AP008984.1	9	284305 284272 284382	$(AGTGAGCAG)_5$ $(AGTGAGCAG)_7$ $(AGTGAGCAG)_4$
DQ657948.1 DQ177346.1 AP008984.1	3	288178 288150 288292	$(TCA)_5$ $(TCA)_5$ $(TCA)_8$
DQ657948.1 DQ177346.1 AP008984.1	2	288776 288749 288890	$(TG)_{46}$ $(TG)_{56}$ $(TG)_{34}$

VNTR	Primer	Sequence	Repeat	Genome Location*	Allele no	No of Repeats
VNTR-11700	11700 For	CACATCATCAAGAACTTCAG	AGTGAGCAG	11628	4	1-6
	11700 Rev	TTGCAGTATTGGAGCACTC				
VNTR-91700	91700 For	GTATGGGTCTAGATAGAGAG	AACCACCGAGG(A/T)	90974	12	0-29
	91700 Rev	GAAGGACCTGACCAACTCAG				
VNTR-133920	133920 For	CTCGCAGATCAGAGGTTCG	ACCCTC	133275	6	5-10
	133920 Rev	GACCTACCTACCTCTACAC				
VNTR-188700	188700 For	CGGAATCCACCACGTACAG	TTC	188012	14	13-26
	188700 Rev	TGAAATCCATCACCTGCGAG				



Dendrogram for 4 VNTR loci, VNTR-91700, VNTR-11700, VNTR-133920 and VNTR-188700

Vaccine-like **Asian** **Intermediate** **European**

VNTR profiles for CyHV-3 detected in the UK

- Approx. 10% of the total number of samples analysed to date
- A total of 75 distinct VNTR profiles were observed for the CyHV-3 detected in the UK between 2003 and 2016, suggesting large numbers of independent introductions
- Highlights the potential for even greater genetic diversity of the CyHV-3 strains circulating globally.

Stability of the VNTR profiles

- The same VNTR profile was observed in all viruses identified during a single disease episode on a fishery site indicating that they stable in the short term and represent suitable markers for epidemiological studies and outbreak tracing.
- Research undertaken at Cefas has demonstrated this experimentally. Virus was reactivated by stressing fish that had survived a KHVD outbreak three years earlier, and the infection was transmitted to naïve cohabiting fish. The virus detected in the stressed animals and that transmitted to the naïve fish was identical to those of the initial outbreak virus

The same VNTR profile was observed in viruses isolated from a fishery site after long periods without clinical disease indicating that the virus can remain dormant for several years, and raises concerns that the virus may be more widespread than originally thought.

9		
J375 (2006)	R135 (2013)	R136 (2013)
5	5	5
25	25	25
5	5	5
14	14	14
Lavender Hall - reoccurance		

24	
S118 (2014)	D182 2.4 (2001)
5	5
18	18
7	7
18	18
linked 13 year gap	

The same VNTR profile was observed on several sites across the country either in the same year or within a year of each other suggesting spread or infected fish from the same supplier

17				
J286 1.1 (2006)	H329 1.1 (2005)	H361 2.1 (2005)	H361 2.2 (2005)	H361 2.3 (2005)
5	5	5	5	5
19	19	19	19	19
7	7	7	7	7
13	13	13	13	13
Linked?				

The majority of the VNYR profiles were unique and found on a single site only suggesting multiple independent introductions.

42	43	44	45	46	47
L151 (2008)	L160 (2008)	DQ177346.1	N099 (2010)	N100 (2010)	P123 1.1 (2011)
5	5	7	5	5	5
18	18	18	18	18	18
9	8	8	7	7	7
19	21	28	19	20	30

KHV specific VNTRs identified in the positive samples from 5 disease outbreaks in Essex in 2014.

		Sample				
		S106 Pea Lane	S118 Great Oakley	S124 Valence	S133 Slough House	S134 Puddledock
VNTR	11700	18	18	18	18	18
	91700	5	5	5	5	5
	133920	22	18	24	22	26
	188700	9	7	9	7	10

KHV specific VNTRs identified in the positive samples from disease outbreaks in Essex in 2014.

		Sample				
		S106 Pea Lane	S118 Great Oakley	S124 Valence	S133 Slough House	S134 Puddledock
VNTR	11700	18	18	18	18	18
	91700	5	5	5	5	5
	133920	22	18	24	22	26
	188700	9	7	9	7	10

KHV specific VNTRs identified in the positive samples from disease outbreaks in Essex in 2014.

		Sample				
		S106 Pea Lane	S118 Great Oakley	S124 Valence	S133 Slough House	S134 Puddledock
VNTR	11700	18	18	18	18	18
	91700	5	5	5	5	5
	133920	22	18	24	22	26
	188700	9	7	9	7	10

Further work

Industrial placement student for 2018/19 looking at the suitability of the CyHV pol PCR assay to demonstrate freedom from infection with CvHV-3.

Design and evaluation of a new real-time PCR assay to capture all CyHV-3 including the CyHV-3 variants

Analysis of the VNTR regions for the more recent CyHV-3 samples to identify links between outbreaks.

Molecular Epidemiology of Herpes Viruses of Fish and Shellfish in the UK.

David Stone, Melanie Dodge, Paul Martin, Gareth Wood, Jackie Savage
Lex Hughes, Chris Pond, Jason Mewett, Nick Stinton and Keith Way

Cefas Weymouth Laboratory