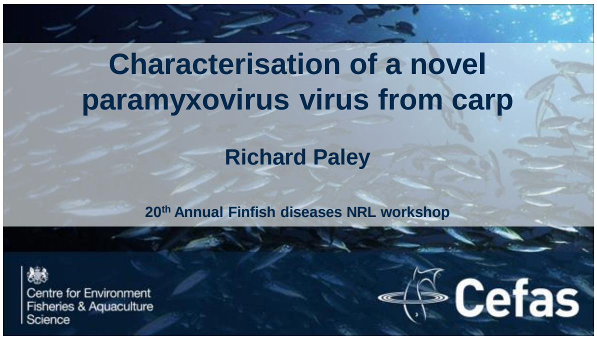


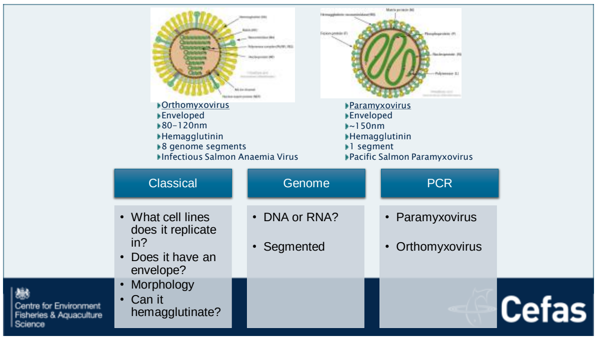
Characterisation of a novel paramyxovirus virus from carp

Richard Paley

20th Annual Finfish diseases NRL workshop

Background

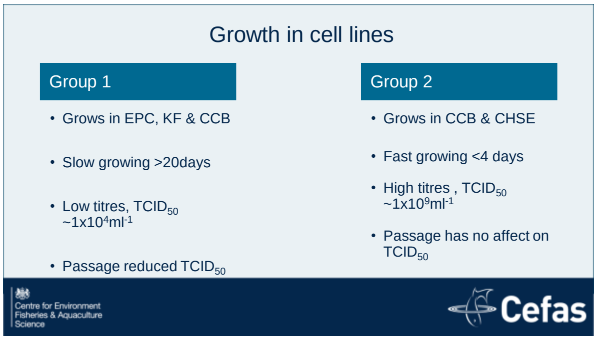
- Syncytium forming cytopathic effect observed in a variety of cyprinids when undertaking screening, routine diagnostics and reported disease outbreak testing for SVCV and KHV
- 22 isolations since 1996
- Limited attempts to characterise generally unsuccessful
- Early EM images show possible myxo-like virus



- Orthomyxovirus
- Enveloped
- 80–120nm
- Hemagglutinin
- 8 genome segments
- Infectious Salmon Anaemia Virus

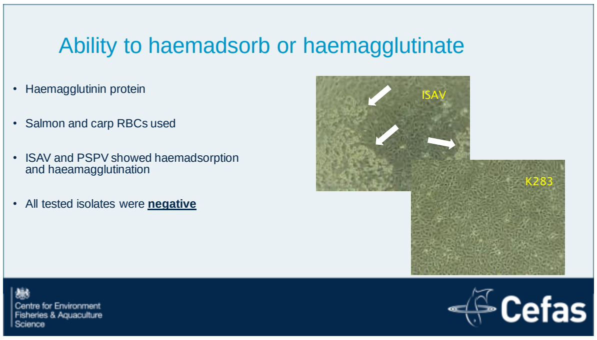
- Paramyxovirus
- Enveloped
- ~150nm
- Hemagglutinin
- 1 segment
- Pacific Salmon Paramyxovirus

Classical	Genome	PCR
• What cell lines does it replicate in? • Does it have an envelope? • Morphology • Can it hemagglutinate?	• DNA or RNA? • Segmented	• Paramyxovirus • Orthomyxovirus



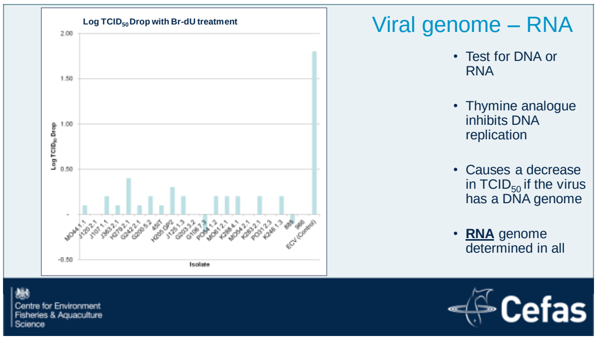
Growth in cell lines

Group 1	Group 2
• Grows in EPC, KF & CCB	• Grows in CCB & CHSE
• Slow growing >20days	• Fast growing <4 days
• Low titres, $TCID_{50}$ ~$1x10^4ml^{-1}$	• High titres , $TCID_{50}$ ~$1x10^9ml^{-1}$
• Passage reduced $TCID_{50}$	• Passage has no affect on $TCID_{50}$



Ability to haemadsorb or haemagglutinate

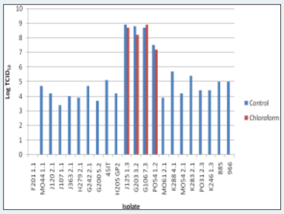
- Haemagglutinin protein
- Salmon and carp RBCs used
- ISAV and PSPV showed haemadsorption and haeamagglutination
- All tested isolates were **negative**



Viral genome – RNA

- Test for DNA or RNA
- Thymine analogue inhibits DNA replication
- Causes a decrease in $TCID_{50}$ if the virus has a DNA genome
- **RNA** genome determined in all

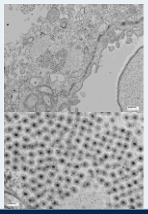
Presence of an envelope

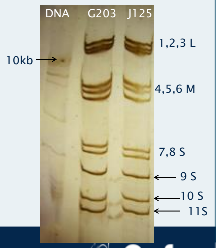


- Chloroform test for lipid envelope
- Causes a drop in $TCID_{50}$ if an envelope is present
- Identified at least 2 different viruses in samples.

4 non enveloped isolates were Aquareovirus - grass carp reovirus

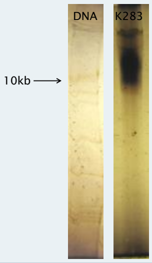
- Non enveloped isolates gave 11 bands= *Aquareovirus*
- *Confirmed by PCR and sequencing*
- *100% id to Grass carp reovirus (polymerase and capsid genes)*





Polyacrylamide gel electrophoresis (PAGE) of viral genome

- Remaining enveloped isolates showed high weight smears
- Not resolved by a longer running time, further phenolic extraction, native or denaturing PAGE



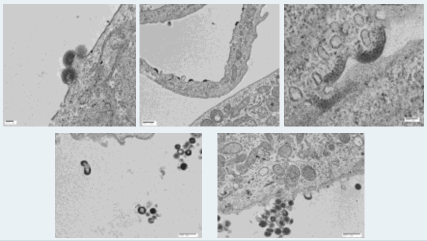
PCR with generic primer sets for Paramyxovirinae and Orthomyxoviridae

- Primers from Tong et. al (2008) targeted ≈700bp L polymerase gene for paramyxovirinae
- Consensus/degenerate hybrid primers (iCODEHOP -University of Washington) for orthomyxo
- Primer sets from Dr William Batts USGS Western Fisheries Research Centre - Isolated various Paramyxoviruses and Orthomyxoviruses from various fish species (unpublished)
- Gradient PCR from 42°-52°C
- ISAV and Pacific salmon paramyxovirus as positive controls

Sensitive and Broadly Reactive Reverse Transcription-PCR Assays To Detect Novel Paramyxoviruses

Test isolates all **negative**

Group 1 – “myxo”-like



TEM

Grp 1- unknown	Grp 2 - Reovirus
myxo-like budding	No budding
Clear membrane	No lipid membrane
Size of ~250nm – possibly a bit large for myxoviruses	Size of ~80nm

Next Generation Sequence analysis

- 2 isolates prepared for NGS
- Clarified and sucrose purified cell culture harvest
- Double stranded cDNA prepared using tagged random primers and reverse transcriptase then sequenase
- Roche 454 (1/8th plate per isolate)
- 6-12k reads per isolate
- CLC genomics for tag removal and *de novo* assembly
- Offline Blast analysis
- Megan, MG RAST, One Codex (Helix I/O)

- No obvious viral sequence, no large contigs
- Host genome, ribosomal RNA, micoplasma contamination in one isolate.

Next Generation Sequence analysis

- Loss of virus during preparation?
  - Confirmed virus presence by EM negative staining
- Insufficient sequencing depth?
  - Repeated one isolate using HiSeq with DNAse and ribodepletion treatment
  - >10m reads
- No significant homology

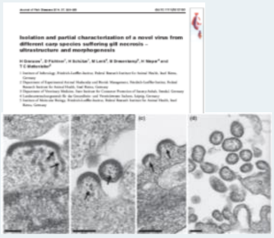
Conclusions

- Isolates are a mix of GCRV and a still unknown virus
- Probably not a myxo-like virus or very distantly related
- Possibly a virus family with similar morphology to *Para/Orthomyxovirus*

Notes

Isolation of Virus-Like Particles From Koi (*Cyprinus carpio*) Suffering Gill Necrosis

A. Body[1], F. Lieffrig[2], G. Charlier[3] & A. Collard[3]



Isolation and partial characterization of a novel virus from different carp species suffering gill necrosis – ultrastructure and morphogenesis

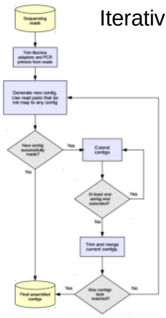
Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality

M. Neukirch and U. Kunz

Enveloped RNA viruses

Family/Genus	Nucleic acid	Configuration	Morphology	Size (nm)	Host
Hypoviridae	ds	1 linear	Pleomorphic vesicles	50-80	Fungi
Cystoviridae	ds	3 linear	Spherical (icos)	85	Bact
Atrerivirus	ss	1 + linear	Spherical	45-60	Vert
Coronaviridae	ss	1 + linear	Roughly Spherical / pleo	120	Vert, (fish)
Flaviviridae	ss	1 + linear	Spherical	50	Vert, Insect
Togaviridae	ss	1 + linear	Spherical (icos)	65-70	Vert, Insect, (fish)
Paramyxoviridae	ss	1 – linear	Spherical	150	Vert, (fish)
Arenaviridae	ss	2 – linear	Spherical	60-300	Vert
Orthomyxoviridae	ss	8 – linear	Spherical	80-120	Vert, (fish)
Bunyaviridae	ss	4-5 +/- linear	Spherical / pleo	80-120	Vert, Insect, Plant
Retroviridae	ss	dimer 1 + linear	Spherical	80-100	Vert (fish)

Iterative Viral Assembler (IVA) pipeline

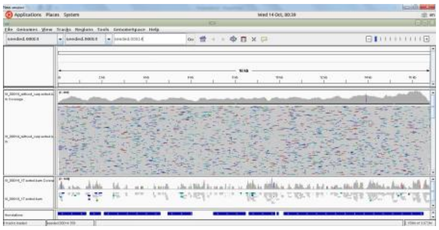


- Designed specifically for read pairs sequenced at highly variable depth from RNA virus samples
- Shown to produce significantly higher quality assemblies than existing approaches
- M061 *de novo* assembly
  ➔ single contig of 16,807bp

Hunt et al 2015 Bioinformatics 31(14):2374-

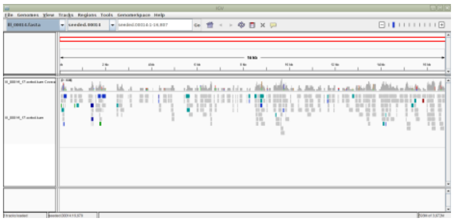
Mapping of reads to the novel virus genome (HiSeq dataset)

	Raw reads	Trimmed paired reads (w/o carp)	Unpaired R1	Unpaired R2	Mapped reads	Percentage	Coverage
M061	10,871,535	6,566,088	78,141	7,071	237,895	1.78%	1854 +/- 761



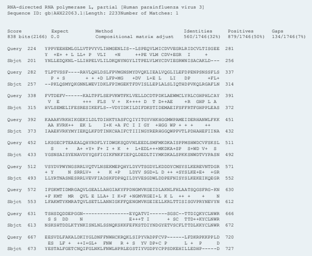
Mapping of reads to the novel virus genome (MiSeq dataset)

	Raw reads	Trimmed paired reads	Unpaired R1	Unpaired R2	Mapped reads	Percentage	Coverage
Sample 16	52,070	48,994	1,820	130	0	0%	-
Sample 17	90,779	86,407	2,294	129	345	0.2%	1.97 +/- 1.98
Sample 18	96,125	92,985	1,617	79	8	0%	-



TAGCACCCA – putative transcriptional start

ACAAAAA – putative poly A initiation site



```
RNA-directed RNA polymerase L, partial [Human parainfluenza virus 3]
Sequence ID: gb|AHX22063.1|Length: 2233Number of Matches: 1

Score           Expect          Method                          Identities      Positives       Gaps
838 bits(2166)  0.0             Compositional matrix adjust     560/1746(32%)   879/1746(50%)   134/1746(7%)

Query  224   YPPVEEHEMLGLLVTPVYVLIHMGENLIS--LSPEQVLMICDVVEGRLRIDCVLTISGEE  281
             Y  +E+ + L LL+ P  VLI   +N     ++PE VLM CDV+EGR  I     +
Sbjct  201   YNLLEDQKNL-LLIHPELVLILDKQNYNGYLITPELVLMYCDVIEGRWNISACAKLD---  256

Query  282   TLPTVSSF----RAVLQHLDSLFPVMGNSMYDVQKLIEALVQGLILEFDPENPSNSSFLS  337
               P + S       + + +D LFP+MG   +DV  L+E L   LI   DP      +FL+
Sbjct  257   --PKLQSMYQKGNNLWEVIDKLFPIMGEKTFDVISLLEPLALSLIQTHDPVKQLRGAFLN  314

Query  338   FVTDEFV------KALTPFLSEPVKWTFKLVELLDCDTPDKLAEWMCLYRLCGHPHLCAY  391
              V  E        +++  FLS  V +  K++++ D  T D++AE    +R  GHP L A
Sbjct  315   HVLSEMELIFESRESIKEFLS--VDYIDKILDIFDKSTIDEMAEIFSFFRTFGHPPLEAS  372

Query  392   KAAAKVRKHIKGEKILDLTDIHKTYASFCQIYITGYVHKHGGMWPAMEIDERHANWLFKK  451
              AA KVRK++  EK L    I+K +A FC I I GY  +HGG WP + + +    ++
Sbjct  373   IAAEKVRKYMYIEKQLKFDTINKCHAIFCTIIINGYRERHGGQWPPVTLPDHAHEFIINA  432

Query  452   LKSGECPTEAEALQHYRDFLYIDWGKSQDVNLEEDLSMFMKDKAISPPMSHWDCVFSKSL  511
               S    +   A+ +Y+ F+ I + K  +  L+EDL+++MKDKA+SP  S+WD V+  S
Sbjct  433   YGSNSAISYENAVDYYQSFIGIKFNKFIEPQLDEDLTIYMKDKALSPKKSNWDTVYPASN  492

Query  512   VSYDVPWYNGSRRLVQTVLNSEKMEPQHYLDYVTSGDYLKDDDYCMSYSLKEREVNTDGR  571
             + Y     N SRRLV+  +   K +P   LDYV SGD+L D ++ +SYSLKE+E+  +GR
Sbjct  493   LLYRTNASNESRRLVEVFIADSKFDPHQILDYVESGDWLDDPEFNISYSLKEKEIKQEGR  552

Query  572   IFGKMTIDMRGAQVLGEALLAHGIAKYFPDNGMVKGEIDLAKNLFHLAATSQGSFRG-KN  630
             +F KMT  MR  QVL E LLA+ I K+F +NGMVKGEI+L K L  ++ +    +    N
Sbjct  553   LFAKMTYKMRATQVLSETLLANNIGKFFQENGMVKGEIELLKRLTTISISGVPRYNEVYN  612

Query  631   TSHSDQDDEPGGN-----------------EYQATVI------SGSC--TTDIQKYCLNWR  666
              S S  DD    N                 E+++T I       + SC  TTD++KYCLNWR
Sbjct  613   NSKSHTDDLKTYNKISNLNLSSNQKSKKFEFKSTDIYNDGYETVSCFLTTDLKKYCLNWR  672

Query  667   EESVDLFAKALDKIYGLDNFFNWHCKRQKLSIPYVADPFCVP------LFDKRPKKPPLD  720
              ES  LF +   ++I+GL+  FNW   R + S  YV DP+C P       L +  P     D
Sbjct  673   YESTALFGETCNQIFGLNKLFNWLHPRLEGSTIYVGDPYCPPSDKEHILLEDHP-----D  727
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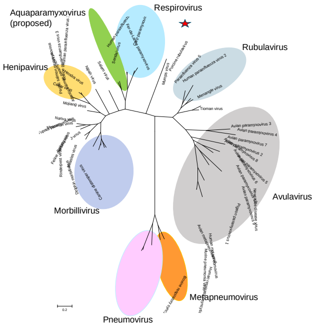
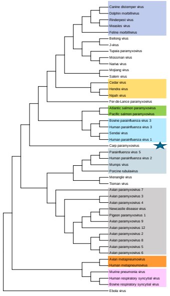


Paramyxoviridae

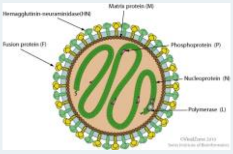


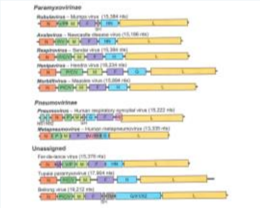
TAGCACCCA – putative transcriptional start

ACAAAAA – putative poly A initiation site

Paramyxoviridae

VIRION - Enveloped, spherical. Diameter of about 150 nm.

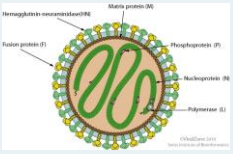


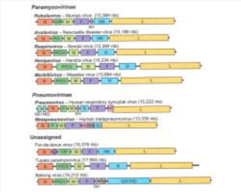


GENOME - Negative-stranded RNA linear genome, about 15 kb in size. Encodes for eight proteins.

Aquaparamyxovirus

VIRION - Enveloped, spherical. Diameter of about 150 nm.
GENOME- Negative-stranded RNA linear genome, about 17 kb in size. Encodes for nine proteins.





Isolate	Host	Origin	Reported diaease	Isolated with
G106	Grass, silver black, bighead carp	Israel	Mortalities	-
F201	mirror/crucian hybrids	-	Disease outbreak	-
G200	Ghost koi	-	NONE	-
G203	Golden Tench	Israel	NONE	-
G242	KOI	UK	Poss bacterial ulcers	-
H205	Common Carp	-	NONE	-
H279	Koi	UK	Lesion behind eye/	Fungal infection
J107	Common Carp	-	NONE	-
J120	Common Carp	UK	Mortalities	CyHV-1
J125	Ghost koi	-	NONE	-
J363	Common Carp	UK	NONE	-
K246	koi/goldfish	UK	Slender, 20% enlarged spleen grey covering on 50% of fish	fungal infection
K283	mirror	-	NONE	-
K288	koi	-	NONE	-
MO44	Common Carp	-	mortalities	Parasites
MO54	Common Carp	-	Similar pathology to Carp pox observed	-
MO61	Common Carp	-	NONE	-
PO31	Roach/common carp	-	Sunken eyes and fin rot	fungal infection
PO54	Koi	-	Mortalities	-
45IT	-	-	-	-
966	-	-	-	-
885	-	-	-	-

Challenge

- IP/cohab in both carp and tench
- 30 fish of each group
- 0.1 ml clarified cell culture harvest (approx. 3.5 TCID$_{50}$/fish)
- Mortalities in IP injected carp only, but up to 43%

Cumulative mortality over 30 days at 17ºC

Isolate	Days post infection									
	3	6	9	12	15	18	21	24	27	30
451									1	1
545								5	7	7
625								2	5	5
669							1	4	8	9
885									3	3
960							2	6	11	13
control	1	1	1	1	1	1	1	1	1	1

Summary

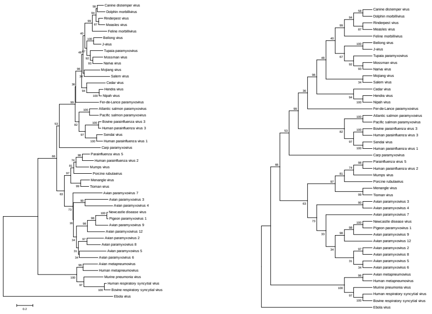
- Finally have identification ……..new virus within the paramyxoviridae - species/genus.
- Characterisation ongoing - genotypic and phenotypic.
- Likely to promote reassessment of the proposed Aquaparamyxovirus - cypriniparamyxovirus.
- Importance of purity of sample, library prep and depth of sequencing.
- Importance of reanalysing data sets and keeping finger on the pulse of new bioinformatics tools.

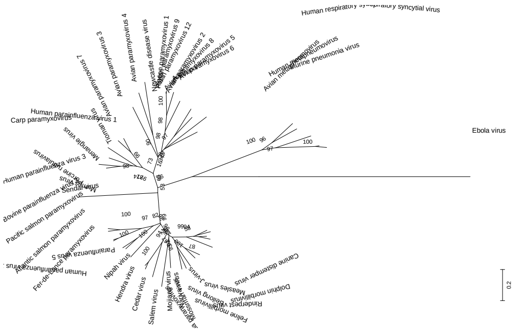
Acknowledgements

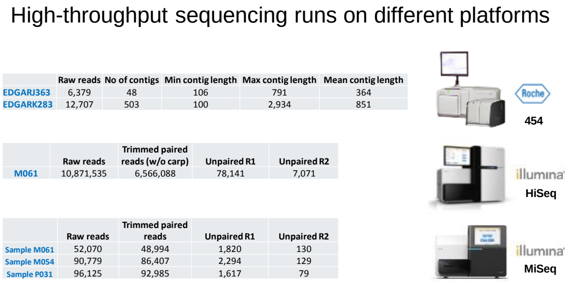
Connor Sharp
Annabel Rice
Kelly Bateman – EM
Irene Cano Cejas – NGS virus prep
Ronnie van Aerle - Bioinformatics
David Stone – molecular virology & bioinformatics
Bill Batts (USGS WFRC) – primer sets & PSPV
Knut Falk (NVI) – ASPV
Defra funding

Centre for Environment Fisheries & Aquaculture Science

Cefas







High-throughput sequencing runs on different platforms

	Raw reads	No of contigs	Min contig length	Max contig length	Mean contig length
EDGARJ363	6,379	48	106	791	364
EDGARK283	12,707	503	100	2,934	851

Roche
454

	Raw reads	Trimmed paired reads (w/o carp)	Unpaired R1	Unpaired R2
M061	10,871,535	6,566,088	78,141	7,071

illumina
HiSeq

	Raw reads	Trimmed paired reads	Unpaired R1	Unpaired R2
Sample M061	52,070	48,994	1,820	130
Sample M054	90,779	86,407	2,294	129
Sample P031	96,125	92,985	1,617	79

illumina
MiSeq