Searching for pathogenic viruses in shellfish using next-generation sequencing

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Outline

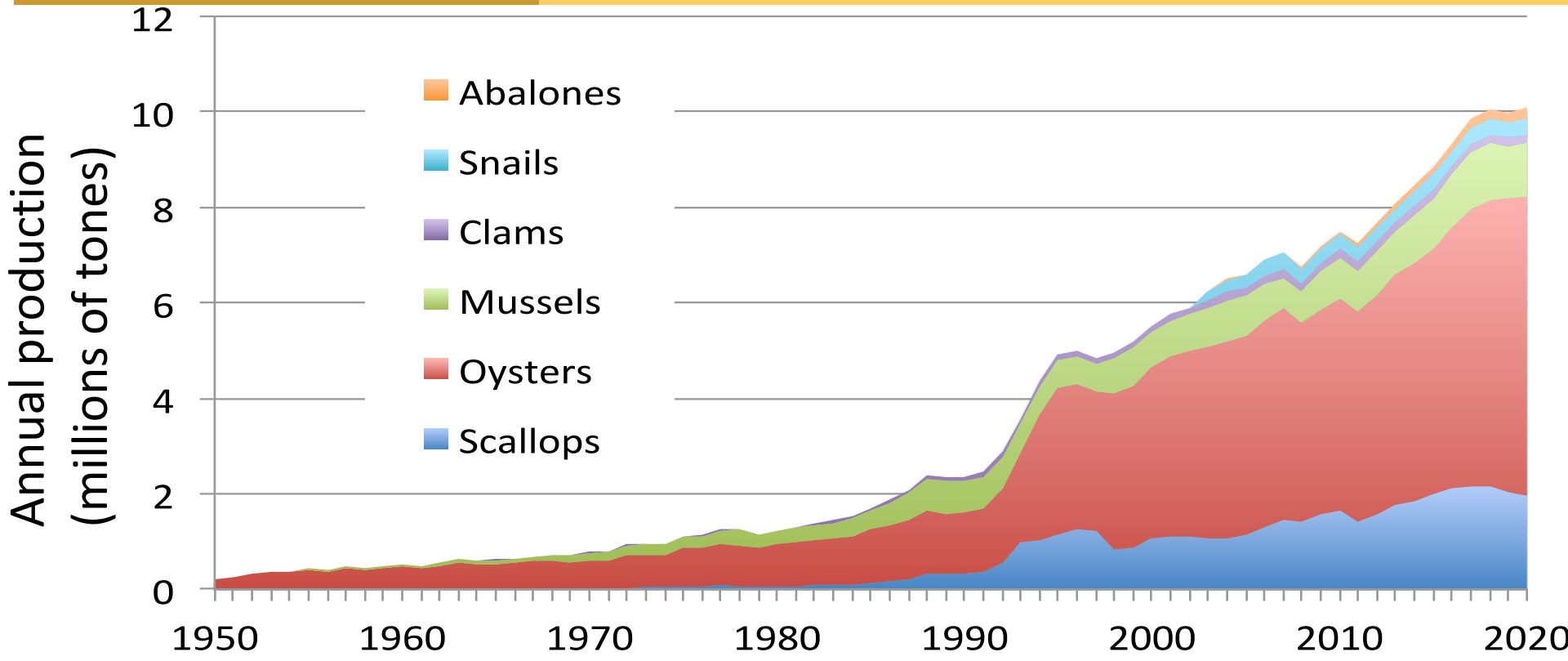
1. The difficulty of identifying viral pathogens in shellfish and the potential of NGS analysis.

2. The use of NGS to identify the pathogen of abalone amyotrophia.

3. NGS search for viruses associated with Pearl oyster (*Pinctada fucata*).

1. The difficulty of identifying viral pathogens in shellfish and the potential of NGS analysis.

Global shellfish aquaculture production (FAO, 2022) continues to increase



- Diseases of larvae and juveniles are not uncommon in shellfish hatcheries, and there are cases of adult shellfish dying in the field.
- Disease is not limited to aquaculture but also occurs in natural populations.
- Often the cause of mortality is unknown.

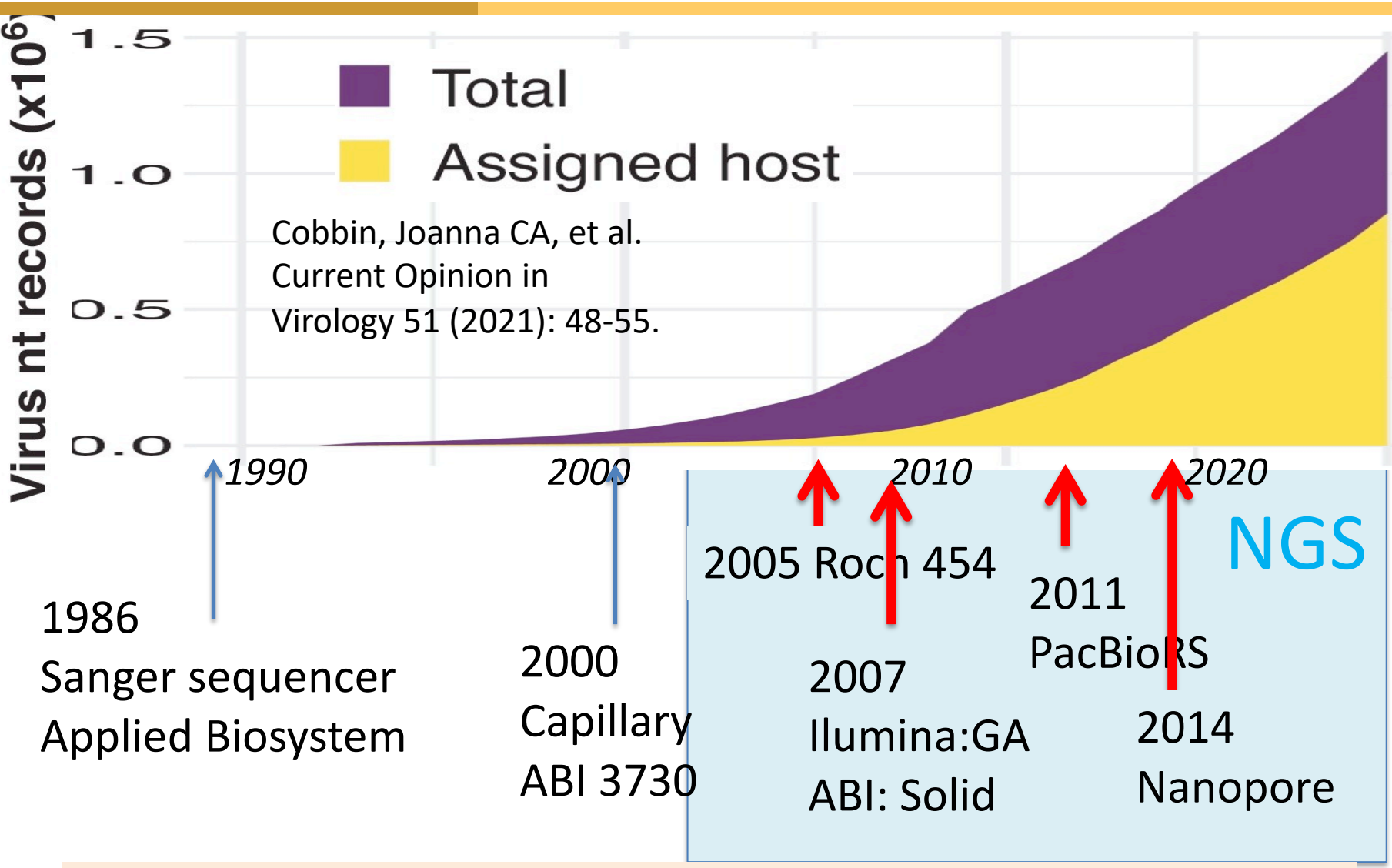
Few pathogenic viruses have been identified

Identifying pathogenic viruses in shellfish is difficult

- There are no cell lines available for virus isolation in shellfish.
- The search for viruses relies on electron microscopy.
- Even if the the virus can be observed by electron microscopy, it does not lead to the molecular approach required for pathogen detection, and does not directly lead to the identification of the pathogen
- Virus-like particles are often observed even in healthy shellfish, morphological observation of viruses alone is not sufficient to identify pathogens.
- To my knowledge, these are the only three viral pathogens for which both viral morphology and the genomic information have been identified.

1. Ostreid herpesvirus (OsHV)
2. Abalone herpesvirus (AbHV)
3. Abalone shriveling syndrome-associated virus (AbSV)

NGS allows comprehensive sequencing without pathogen isolation



Rapid improvements in sequencing technology have led to the accelerated discovery of a novel viruses.

2.
NGS analysis to identify the pathogen of abalone amyotrophia.

The pathogen of abalone amyotrophia is known to be a virus, but the virus had not been identified.

Abalone amyotrophia

- The disease occurred during seed production in the late 1970s.
- Cumulative mortality rates can be as high as 50%.
- Mortality is chronic, and it takes about 1 months from the start of infection until mortality begins.
- The disease occurs at water temperatures around 20℃ and is cured at temperatures above 25℃.
- Can be controlled by UV irradiation of water supply.

Characteristics of the pathogen of abalone amyotrophia

- Size of pathogen: 100~220 nm, confirmed by infection tests using filtrate.

- Inactivated by chloroform: enveloped virus.

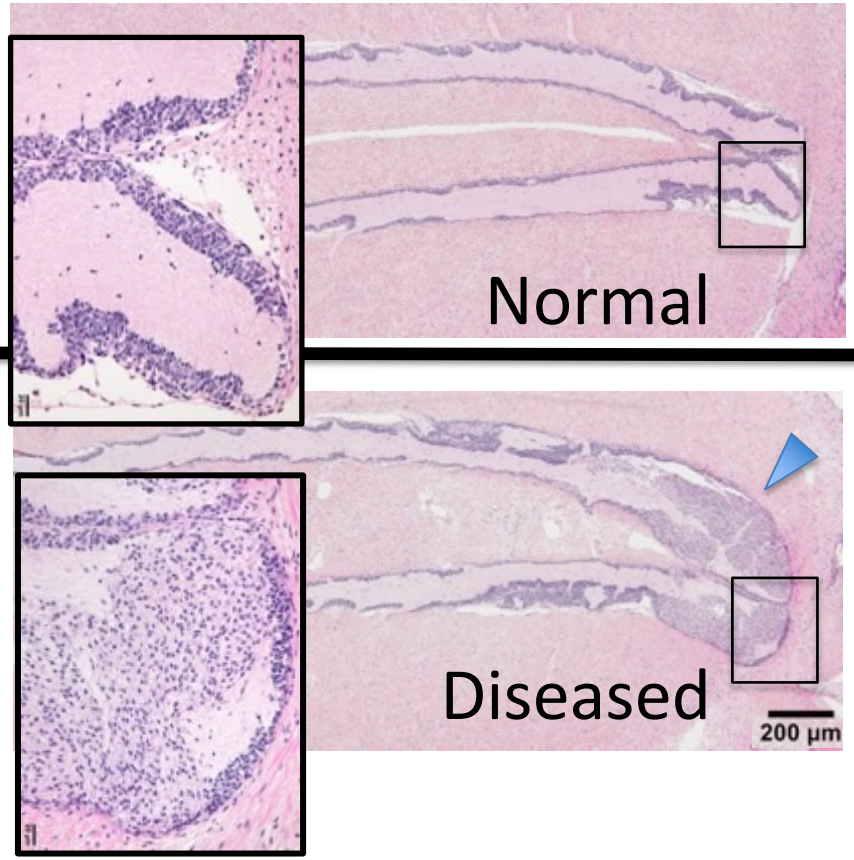
- Tissues from diseased abalone have been screened for pathogenic viruses by electron microscopy, but those studies were not successful.

External appearance and histopathological characteristics Black abalone (*H. discus discus*)

Normal

Diseased

Notches on the front margin of the shells (arrowheads) and brown pigmentation inside the shell (arrows)



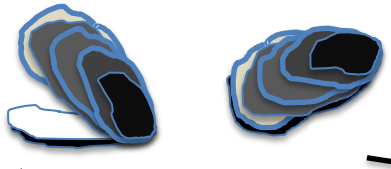
Abnormal cell masses are observed around the ganglion (nerves).

We attempted to identify pathogenic virus using NGS.

Typical NGS strategies for virus detection

- Transcriptome analysis
- Shotgun analysis of purified pathogen

Transcriptome or Shotgun sequence



Sample collection

Transcriptome

Total RNA

All pathogens, including viruses, express RNA. All pathogens can be analyzed using the similar method. No prior information on the pathogen is required.

Disadvantage:
Difficult to detect pathogens that are latent and not multiplying.

Shotgun sequence

Virus purification

DNA/RNA extraction

Genome of the purified pathogen is analyzed.

Effective when some characteristics of the pathogen, such as purification conditions are known.

Library Preparation

NGS

Select disease specific sequences

Taxonomic profiling

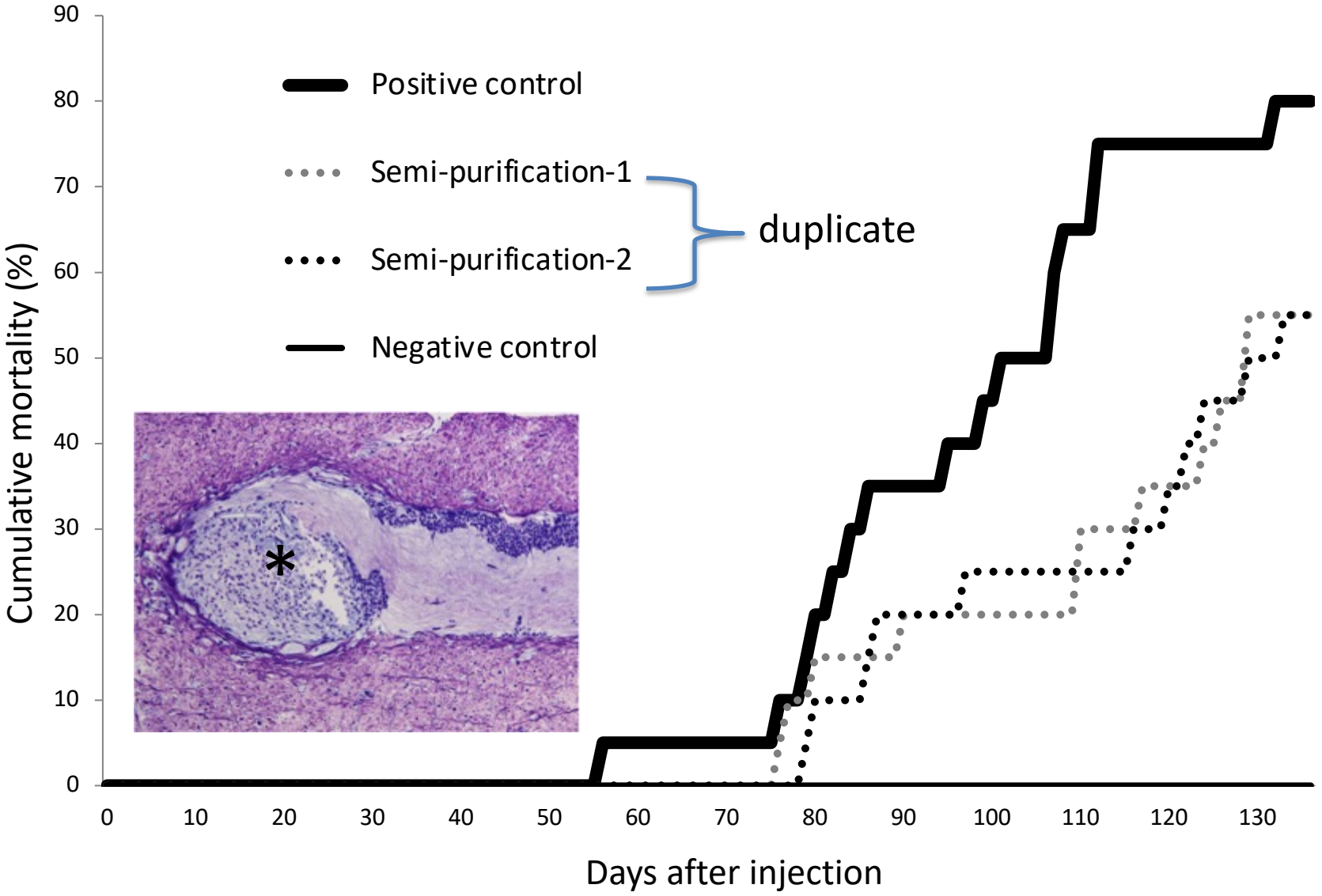
The shotgun sequence was chosen because some nature of the abalone virus was already reported.

Shotgun sequencing analysis is prevented by host-derived DNA/RNA.

Semi-purification of causative agent

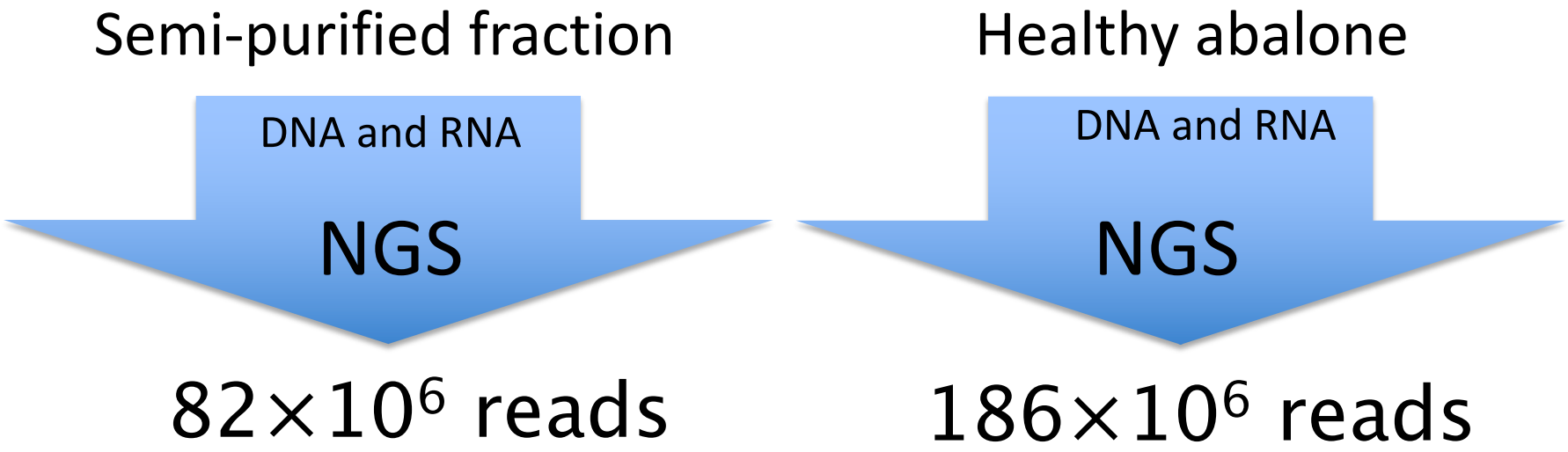
1. Diseased abalone was homogenized and filtrated 0.22um.
2. Abalone-derived nucleic acids were digested with DNase and RNase .
3. Pelleting by ultracentrifugation (150,000 × g, 1 h) .
4. Sediment was resuspended in Sea water.
5. The existence of the pathogen in the suspension was confirmed by infection test.

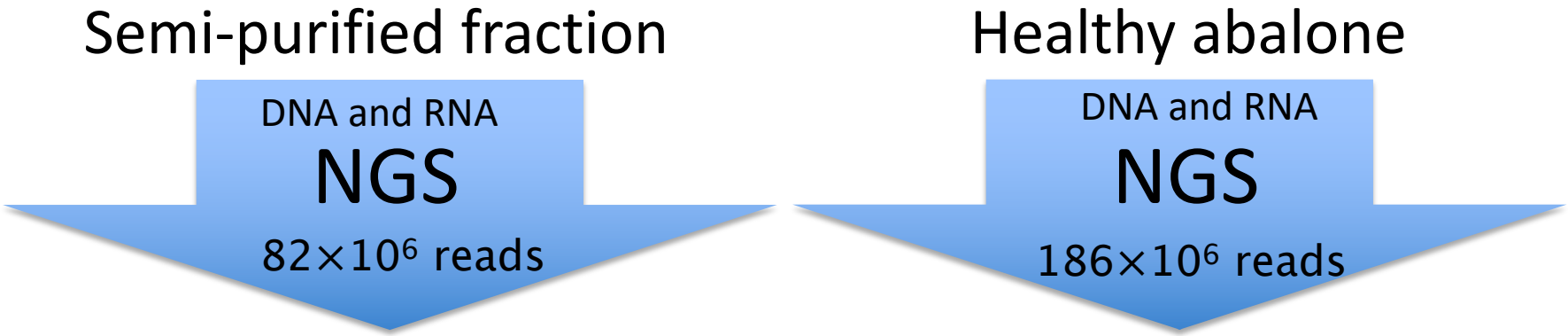
Results of infection testing of crude purified fraction



NGS analysis of semi-purified fraction

- DNA and RNA extracted from the purified fraction and healthy abalone were sequenced by NGS separately.
- Healthy abalone sequencing was used to remove abalone-derived sequences from purified fraction data in silico.





1. Assembly was performed after pooling the reads from the semi-purified fraction and those from healthy abalone. (3237 scaffolds)

(Scaffold is a long sequence formed by assembling reads.)

2. Scaffolds shorter than 500 bp were removed. (149 scaffolds remained)

3. Extracted scaffolds consisted only of reads obtained from the semi-purified fraction. (69 scaffolds)

4. Subjected to BLASTN analysis and removed the scaffolds showed homology to the abalone sequence. (10 scaffolds were removed)

5. Remaining 59 scaffolds were subjected to epidemiological survey.

PCR- and RT-PCR-based epidemiological surveys

Further screening of sequences specific to diseased abalone.

- Specific primers were designed for each of the 59 Scaffolds.
- Epidemiological studies were conducted on healthy and diseased abalone using PCR and RT-PCR to find Scaffolds specific for diseased abalone.

<Analyzed Samples>

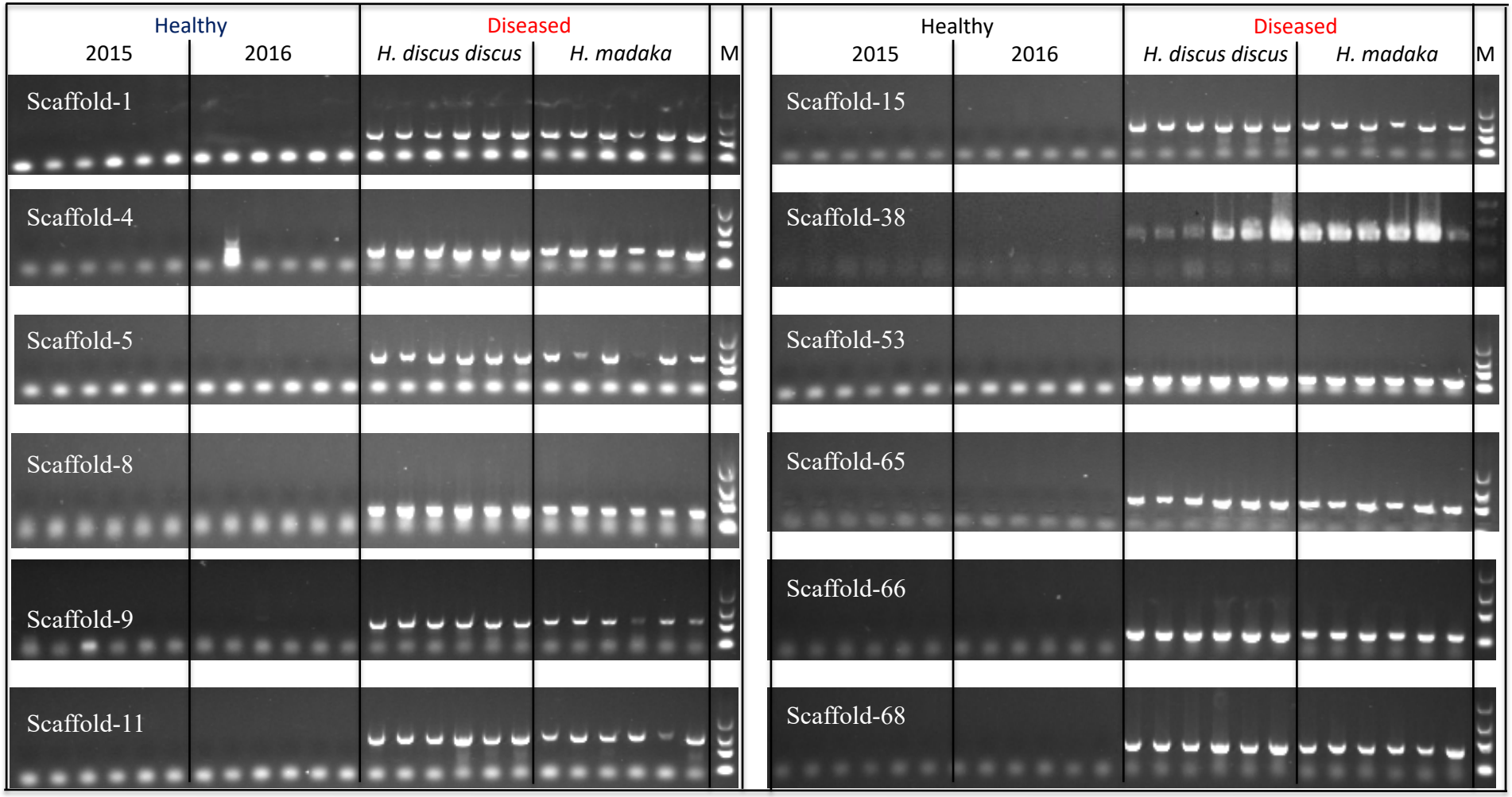
Healthy abalones: *H. discus discus* 2015 and 2016

Diseased abalones: *H. discus discus* 2015, *H. madaka* 2016

N=6 per group.

Results of PCR and RT-PCR -based epidemiological survey

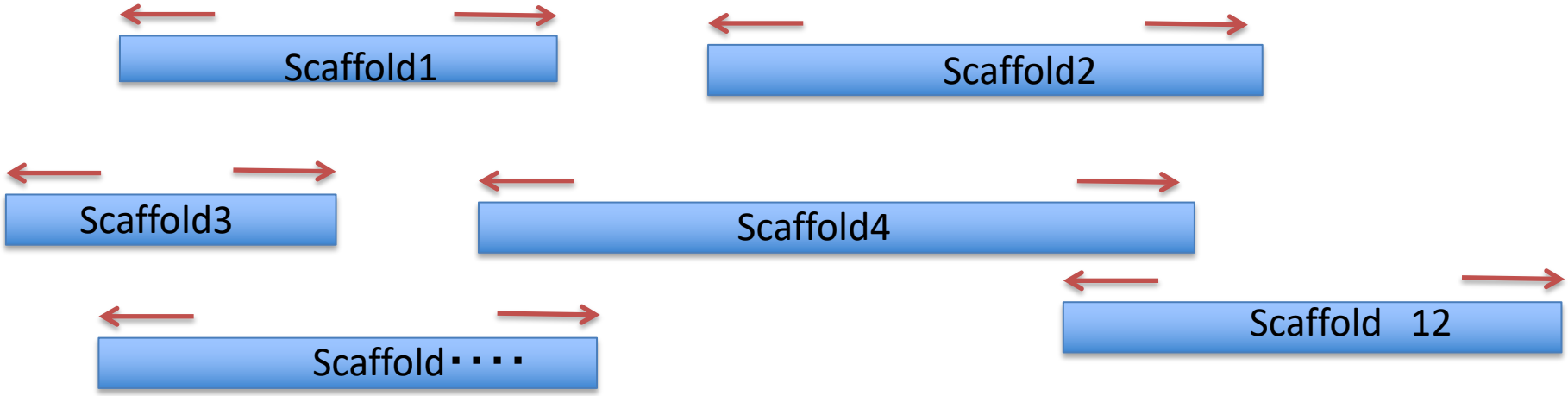
Of the 59 Scaffolds selected by NGS analysis, 12 were positive in all diseased abalones and negative in all healthy.



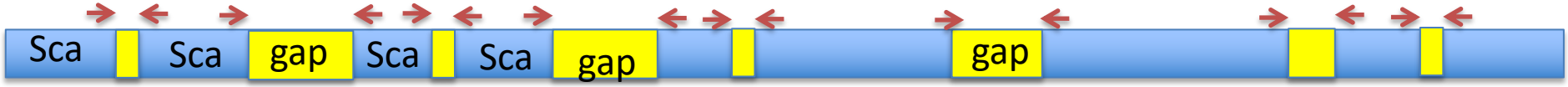
Are all these 12 scaffolds sequences from a single DNA virus?
All 12 Scaffolds consisted of DNA sequence reads and were detected in diseased shellfish by PCR.

Gap-closing PCR

primers were created in the outward direction at the end of each scaffold.
All combinations, 12 x 12 PCRs, were performed.



Amplicons were obtained between 10 of 12 Scaffolds



155,181bp

Predicted coding sequences : 159

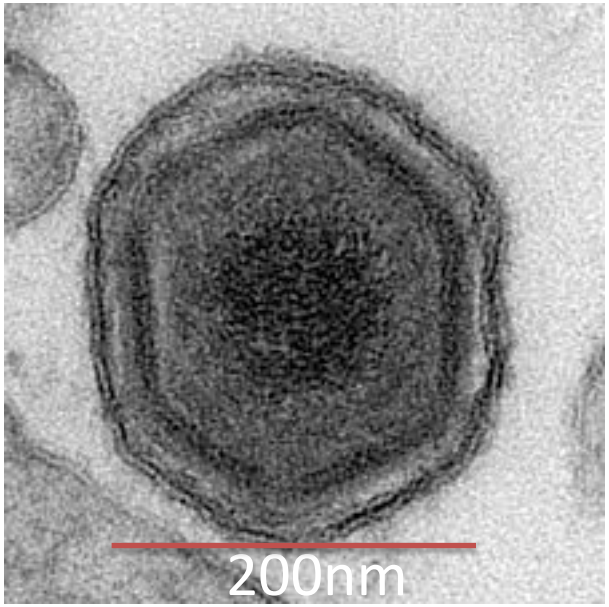
Blast analysis 159 predicted genes

47 genes are homologous to ASFV genes

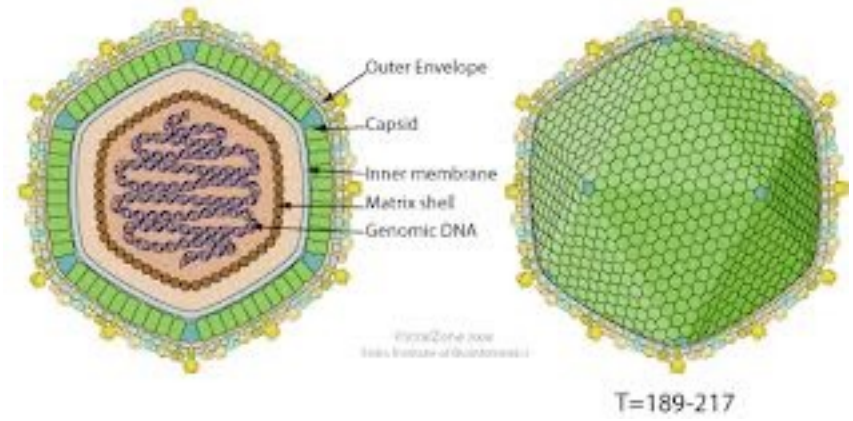
Family	Number of top-hit ORFs
Asfaviridae (Affrican swine fever virus)	47
Asfaviridae ? (Faustovirus and Pacmanvirus)	6
Mimiviridae	2
Pithoviridae	1
Poxvirus	1
Phycodnaviridae	1
Baculoviridae	1
Myoviridae	1
Siphoviridae	1
no hit	98
Total	159

A virus closely related to African swine fever virus is predicted to be a pathogen of abalone.

What African swine fever virus (ASFV) is ?



https://vet.purdue.edu/addl/news/180828-ASF-BOAH-Veterinary-Advisory.php

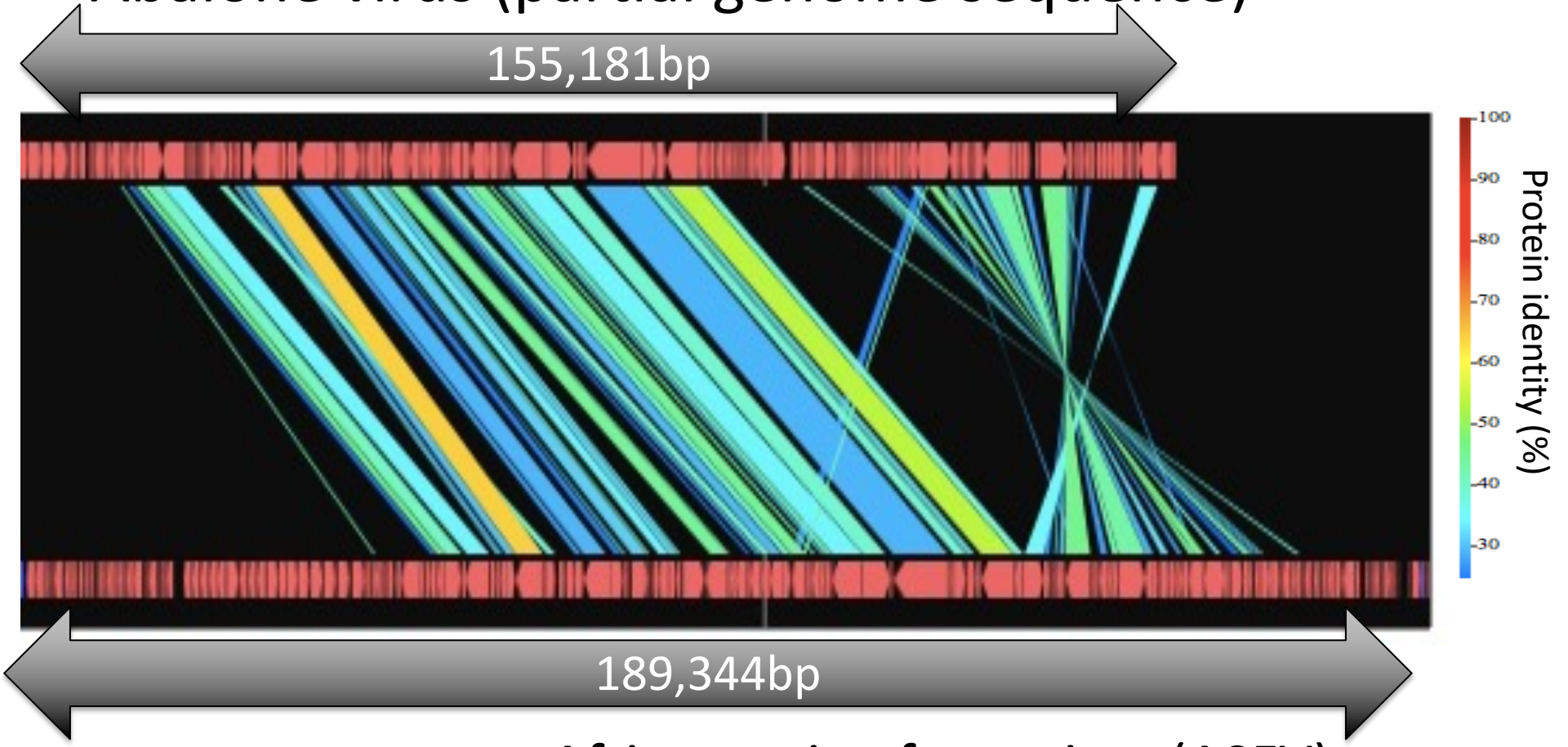


https://viralzone.expasy.org/12

Highly pathogenic in swine (mortality rate ~100%)
Multiplies mainly in macrophages
Nucleo Cytoplasmic Large DNA Virus (NCLDV)
Sole member family Asfarviridae

Synteny analysis

Abalone virus (partial genome sequence)



African swine fever virus (ASFV)

Genome structure is conserved in two viruses

SCIENTIFIC REPORTS
nature research

There are amendments to this paper

OPEN

A novel Asfarvirus-like virus identified as a potential cause of mass mortality of abalone

Tomomasa Matsuyama[1*], Tomokazu Takano[1], Issei Nishiki[2], Atushi Fujiwara[3], Ikunari Kiryu[4], Mari Inada[4], Takamitsu Sakai[1], Sachiko Terashima[1], Yuta Matsuura[1], Kiyoshi Isowa[5] & Chihaya Nakayasu[1]

Abalone asfa-like virus (AbALV) was proposed as the provisional name of pathogen.

Histopathology

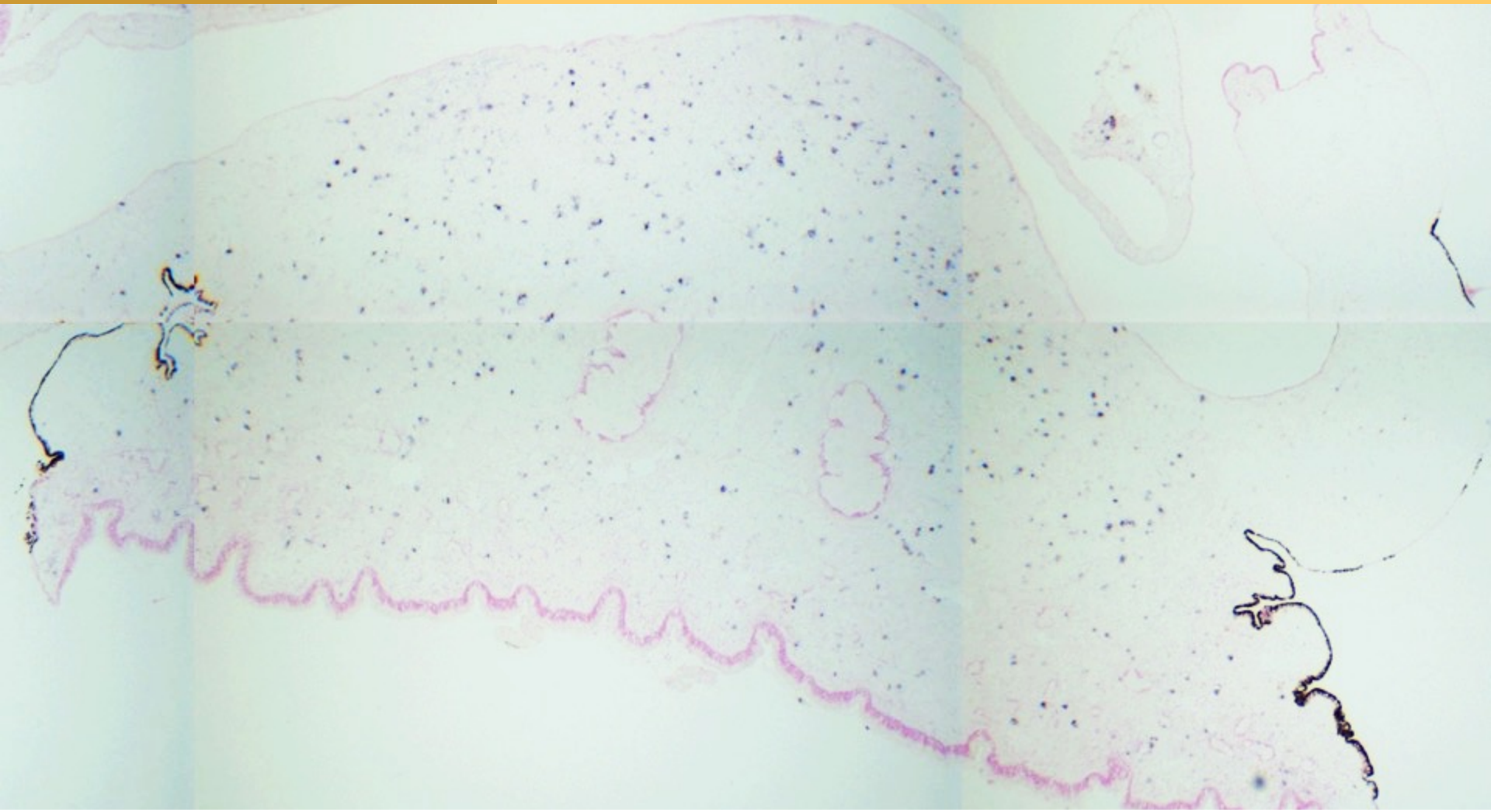
- In situ hybridization

Dig-labelled probes for the Major capsid protein (MCP) gene.

- Immunostaining

Mouse antiserum to *E. coli* recombinant protein of MCP

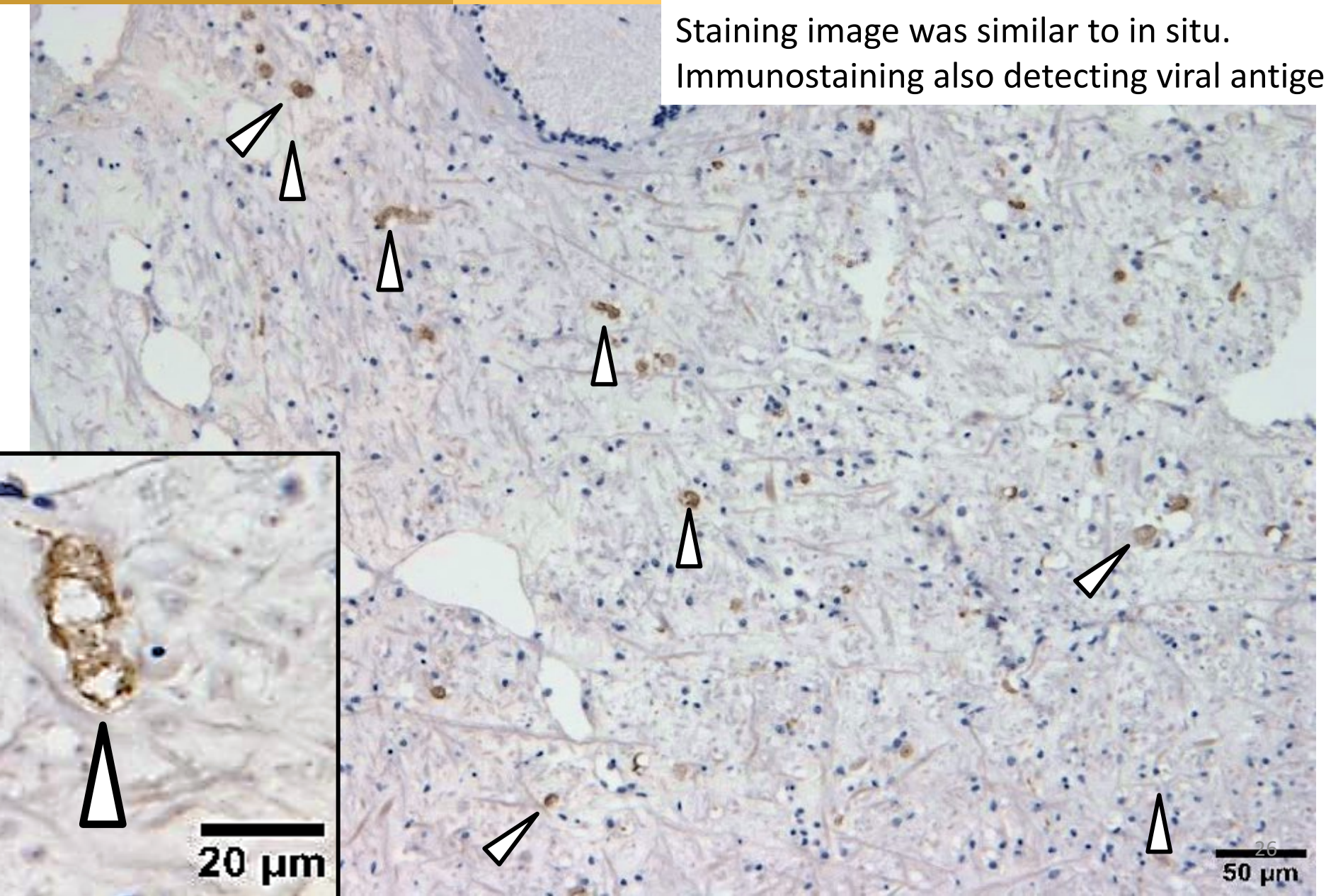
In situ hybridization



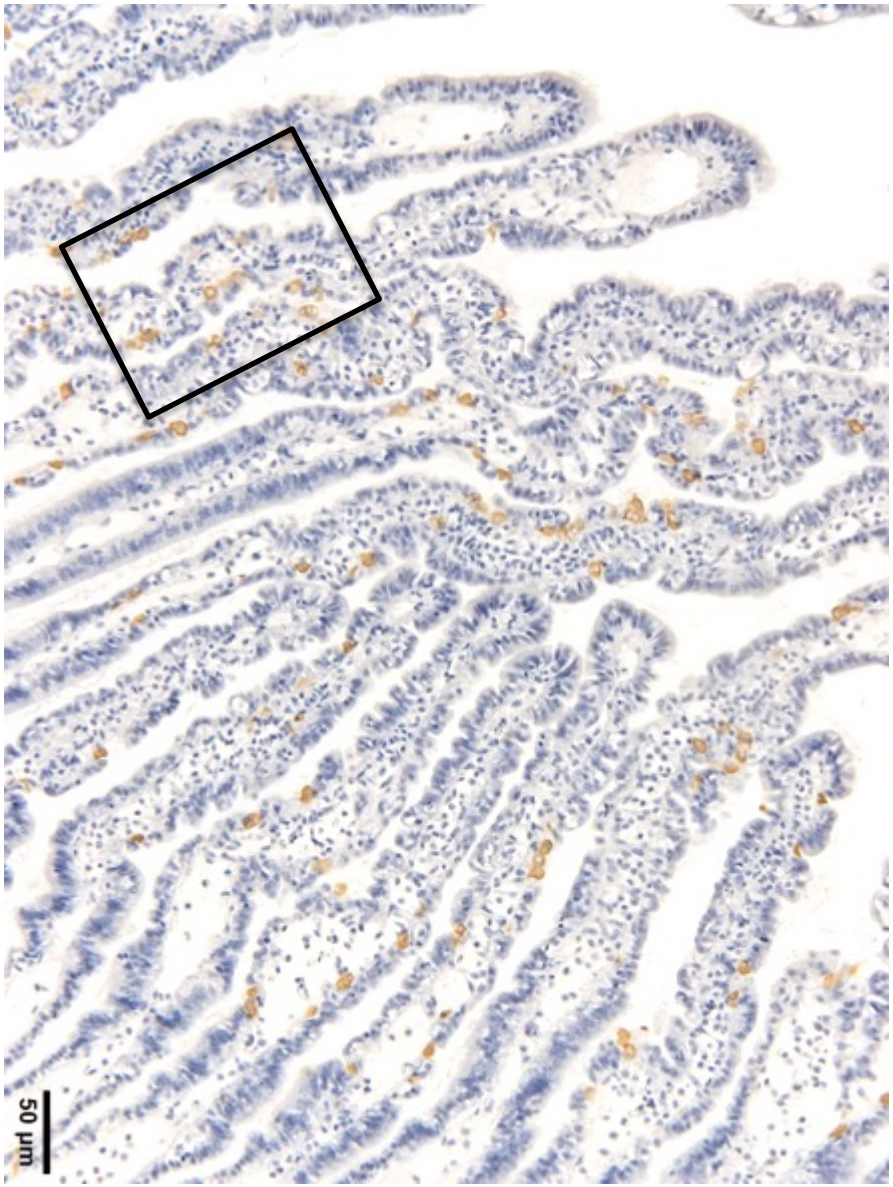
Positive signals scattered throughout the muscle.

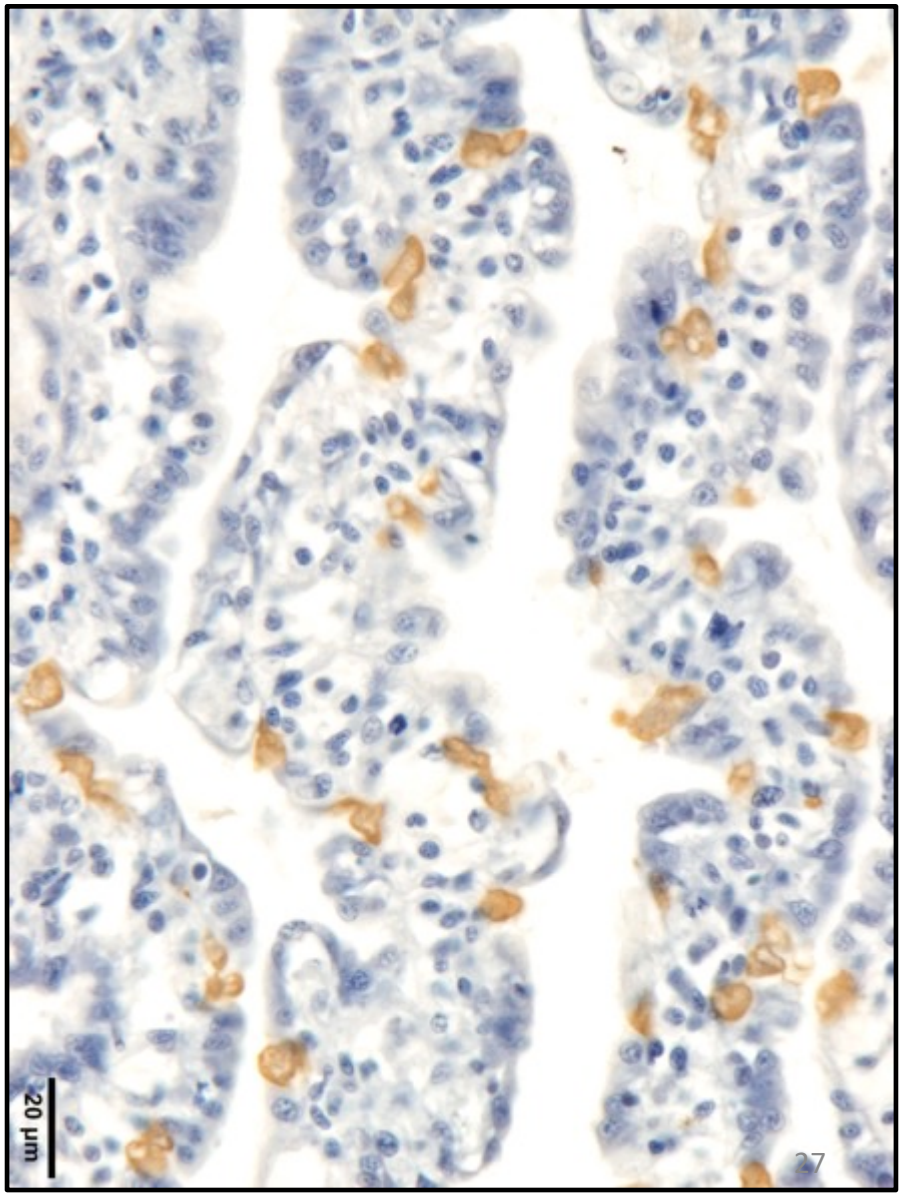
Immunostaining (same paraffin block as ISH)

Staining image was similar to in situ.
Immunostaining also detecting viral antigen.



Positive reactions were most frequently observed on gill surface cells.





Virus dynamics in infection test was investigated by quantitative PCR and immunostaining.

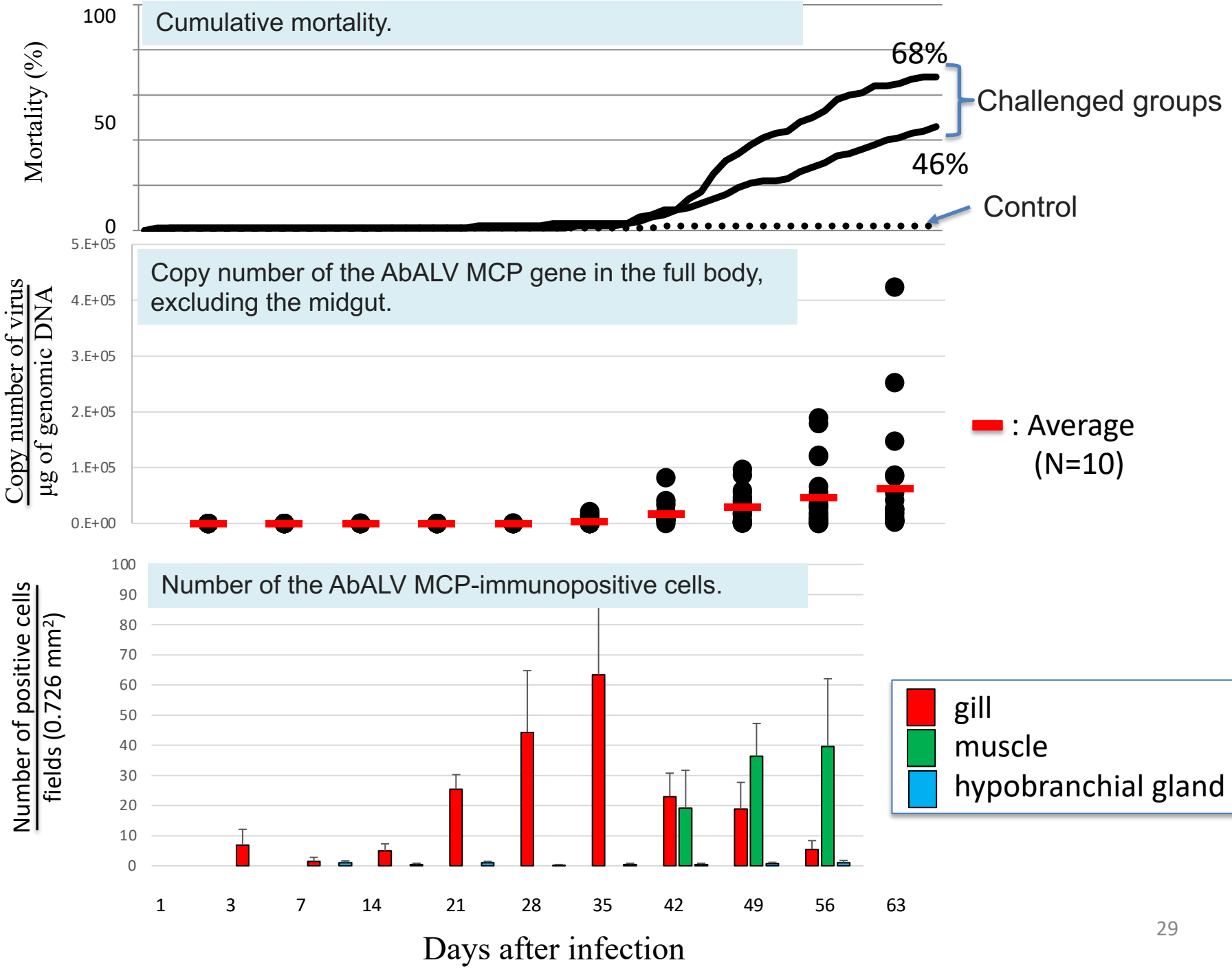
Effluent from infected Abalone (*H. gigantea,* N=10) was continuously supplied for 3 days.

- Naïve abalone (*H. gigantea):* 7-month-old

  Observation groups：N＝100×2 tanks

  Sampling group: N=500×1 tank

Water temperature: 18-20 ℃


Cumulative mortality.
Mortality (%)
100
50
0
68%
46%
Challenged groups
Control
Copy number of the AbALV MCP gene in the full body, excluding the midgut.
Copy number of virus / μg of genomic DNA
5.E+05
4.E+05
3.E+05
2.E+05
1.E+05
0.E+00
: Average (N=10)
Number of the AbALV MCP-immunopositive cells.
Number of positive cells / fields (0.726 mm²)
100
90
80
70
60
50
40
30
20
10
0
gill
muscle
hypobranchial gland
1
3
7
14
21
28
35
42
49
56
63
Days after infection

Although Koch‘s postulates have not been satisfied, the causative agent of abalone amyotrophia was identified.

Can we find pathogens in bivalves by the same way?

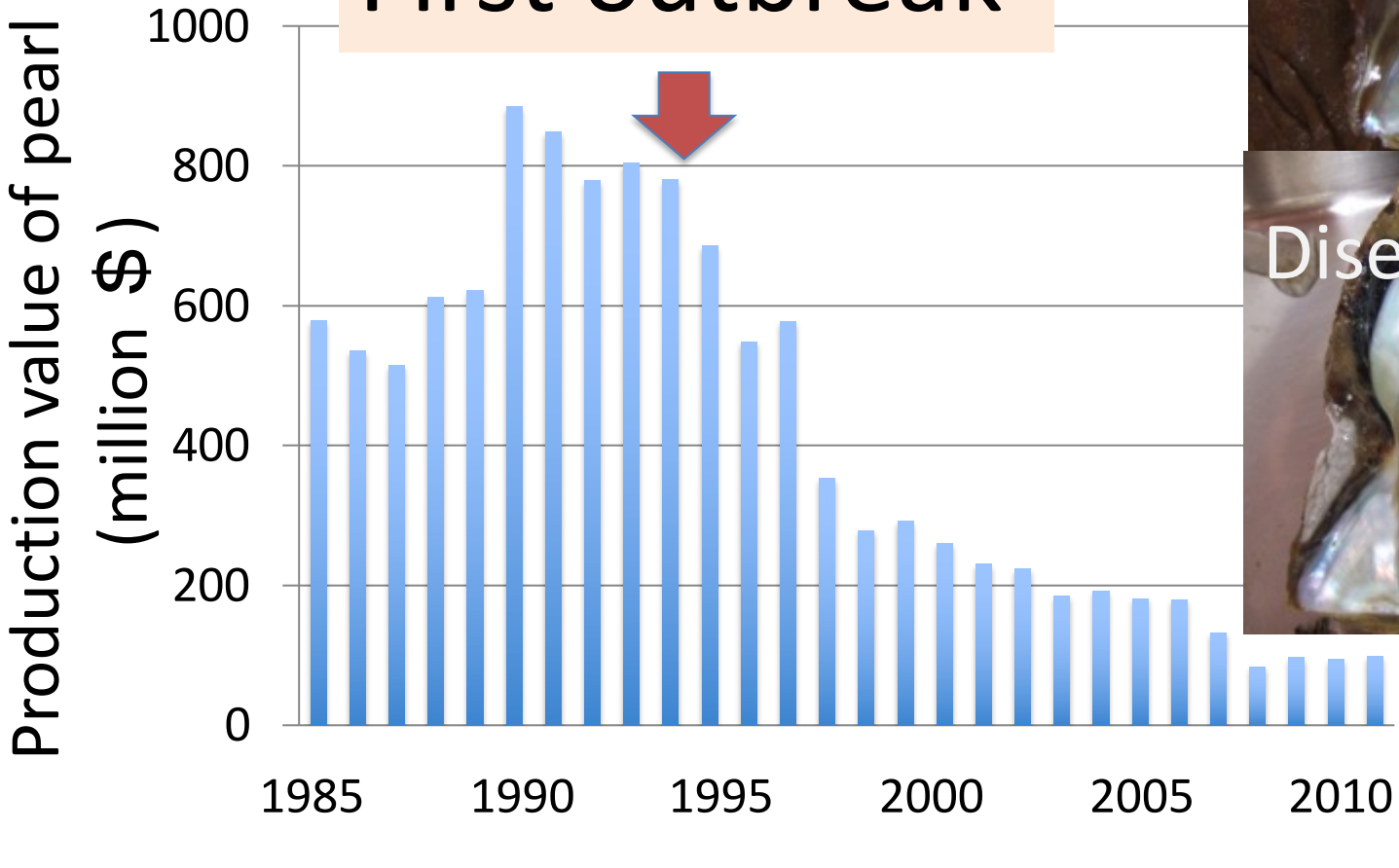
In bivalves, finding pathogenic virus by shotgun sequencing is more difficult than gastropods.

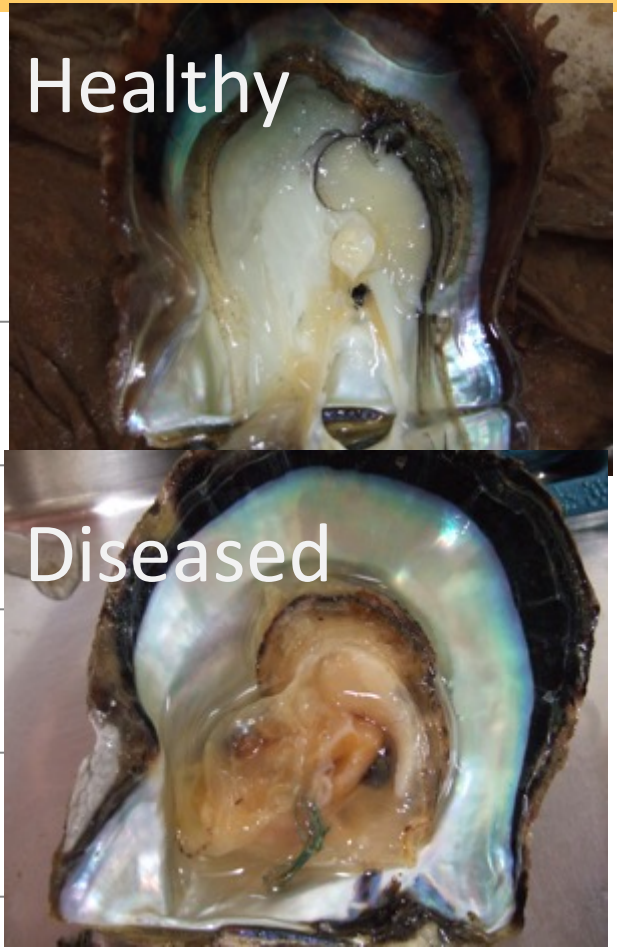
This is because much more variety of viruses can be detected in bivalves than gastropods.

As an example, I will present our analysis of Akoya oyster disease (AOD) in pearl oyster (Pinctada fucata).

3. Akoya oyster disease

A mass mortality event in 1994 greatly reduced the quantity of Akoya pearl.

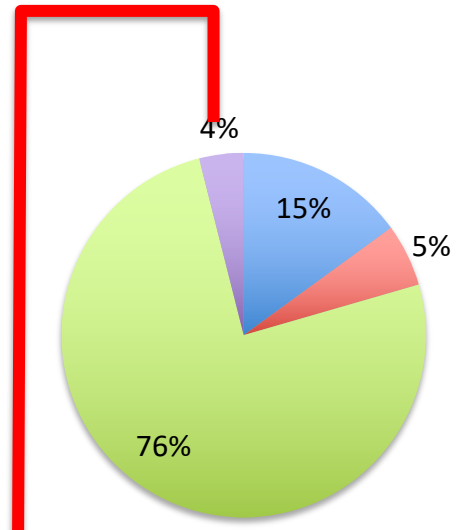




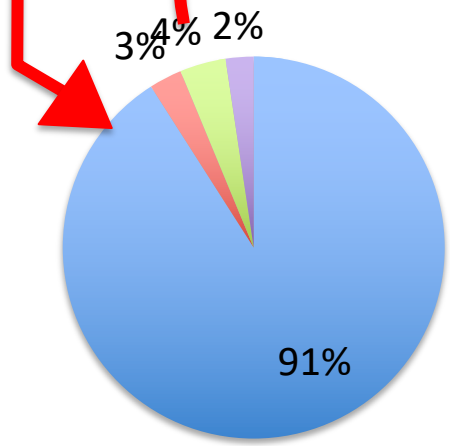
Adductor muscle and mantle turn a reddish-brown color

We performed NGS analysis to identify the pathogen.

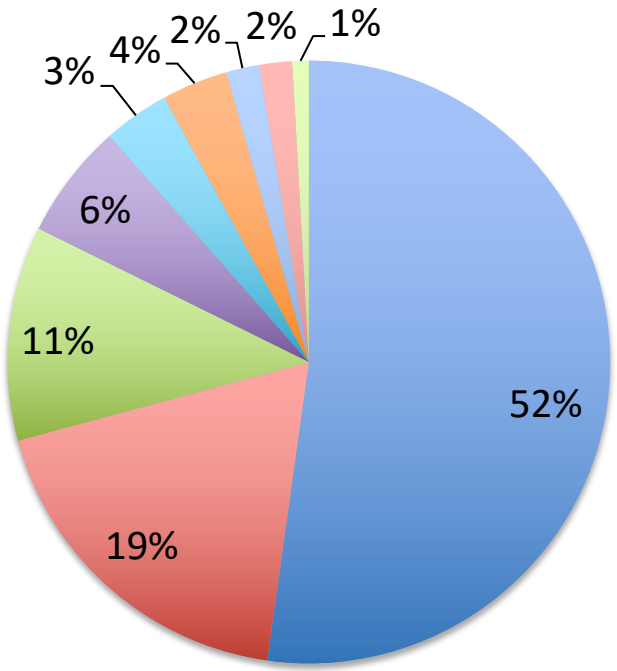
Shotgun sequence of semi-purified fraction of hemolymph (centrifugal sediment: 20,000 × g 60min)



Eukaryote
Archaea
Bacteria
Virus



Virus Host
Bacteria
Algae
Animal
Un known



Animal viruses
Flaviviridae (ssRNA)
Mimiviridae (dsDNA)
Megaviridae (dsDNA)
Herpesviridae (dsDNA)
Circoviridae (ssDNA)
Baculoviridae (dsDNA)
Retroviridae (ssRNA)
Poxviridae (dsDNA)
Polydnaviridae (dsDNA)

huge variety of viruses were detected

shotgun sequence detected huge variety of viruses

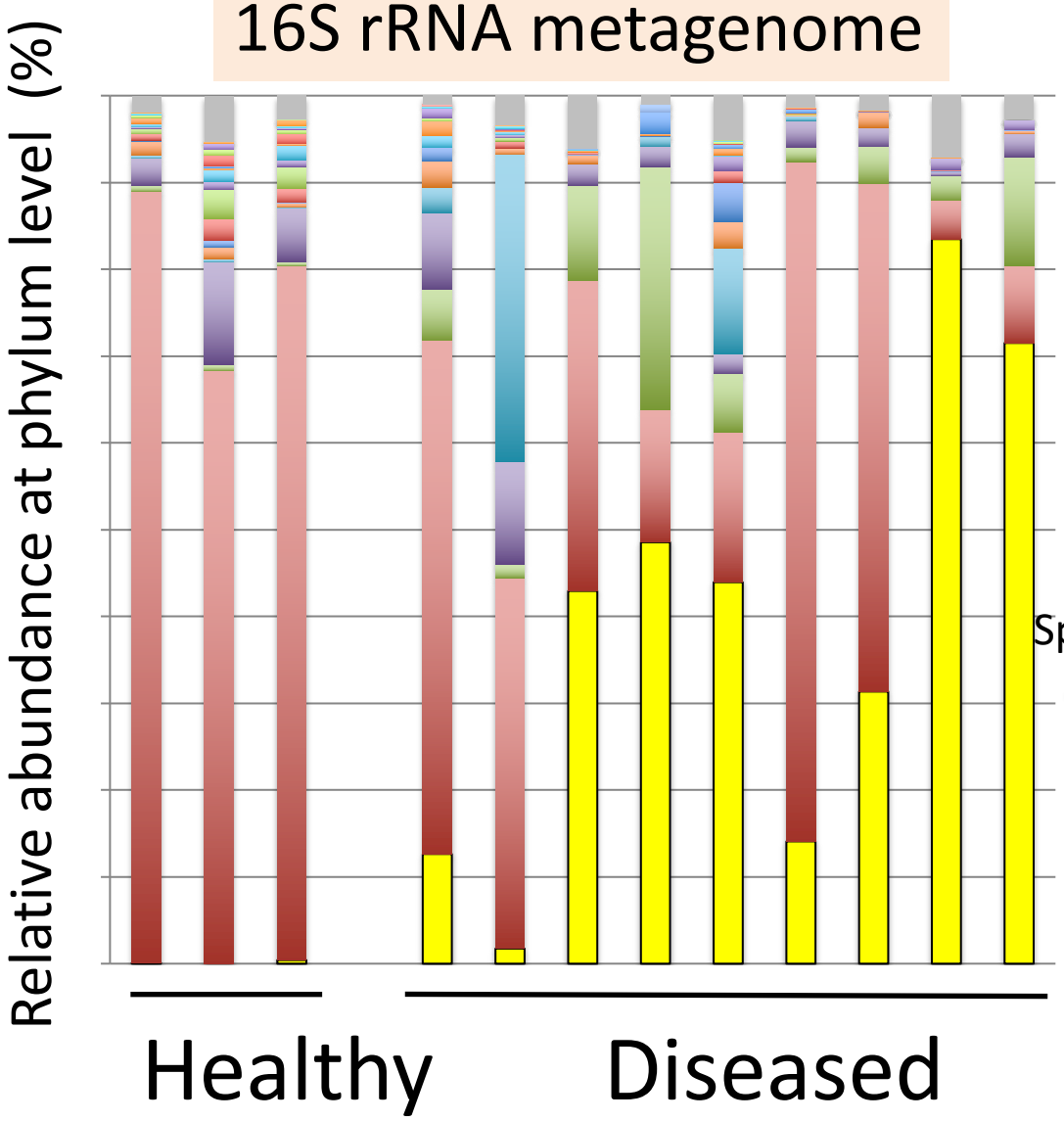
- Filter feeder bivalves filter large amounts of water and capture particles.

- Bivalves may directly capture viruses, or NGS may detect viruses infecting bacteria or micro-organisms that have been captured by shellfish.

- Comparative analysis with healthy shellfish is required to find viruses specific to diseased individual. Alternatively, transcriptome analysis may be more suitable in bivalves.

Causative agent of AOD is a Spirochaeta

16S rRNA metagenome

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RESEARCH ARTICLE

A Spirochaete is suggested as the causative agent of Akoya oyster disease by metagenomic analysis

Tomomasa Matsuyama, Motoshige Yasuike, Atushi Fujiwara, Yoji Nakamura, Tomokazu Takano, Takeshi Takeuchi, Noriyuki Satoh, Yoshikazu Adachi, Yasushi Tsuchihashi, Hideo Aoki, Kazushi Odawara, Shunsuke Iwanaga, Jun Kurita,

ELSEVIER

Aquaculture

Volume 513, 15 November 2019, 734433

Spatiotemporal dynamics of Spirochaeta, the putative etiologic agent of Akoya oyster disease in pearl oysters, as determined by quantitative PCR

Tomomasa Matsuyama [a], Tomokazu Takano [a], Chihaya Nakayasu [a], Kazushi Odawara [b], Yasushi Tsuchihashi [c], Shinji Tanaka [c], Motoshige Yasuike [d], Atushi Fujiwara [d], Yoji Nakamura [d], Tetsuji Masaoka [a]

Spirochaeta

provisional name:
Candidatus Maribrachyspira akoyae

Conclusions

- NGS analysis is suitable for the search for viral pathogens in shellfish.
- However, NGS analysis alone is not enough.
- NGS is useful for screening sequences that may be of pathogen origin. Selected sequences can then be used to detect a pathogen by epidemiological or histopathological analysis.
- No special techniques are required for these analyses.
- I think the most important is to establish a reproducible experimental infection method.
- Using the infection test as a starting point, NGS screening and subsequent epidemiological analysis, etc. can be used to find a pathogen.

Thank you for your attention