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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Ecologie, Evolution, Ressources Génétiques, Paléobiologie

École doctorale GAIA

Institut des Sciences de l'Evolution de Montpellier (ISEM)

Role of structural variations in the genetic diversity and evolution of the large dsDNA Cyprinid herpesvirus 3

Présentée par Nurul Novelia FUANDILA
Le 14 décembre 2023

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Abstract

Evolution of viruses is the result of a tradeoff between genetic diversification and selection. Though it has long been considered that single nucleotide polymorphisms (SNPs) were the main drivers of genetic diversity, it is now acknowledged that structural variations (SVs) may play a great role in virus diversity. The large dsDNA Cyprinid herpesvirus 3 (CyHV-3; 295 kbp) is one of the most pathogenic aquatic viruses. CyHV-3 mainly infects common carp and koi fish and causes mass mortalities, up to 100%. CyHV-3 exists in its host as a population of closely related genetic variants, or haplotypes. Knowledge on the mechanisms and evolutionary consequences of this intra-host diversity is essential in developing preventive strategies such as vaccines.

An earlier study had revealed that the composition of haplotype assemblies may quickly vary along consecutive *in vitro* infectious cycles, resulting in quick phenotypic modifications. Of interest, a deletion of 1363 pb in the ORF150 (ORF150-del) was associated with a loss of virulence. These findings laid the basis of the present work, around three main objectives: 1) get more insights into the evolutionary mechanisms underlying such haplotype assemblies in cell culture, with a focus on ORF150; 2) investigate the prevalence of ORF150-del haplotype and other structural variations in this genomic region *in vivo*; 3) explore the accumulation pattern of SVs and SNPs in CyHV-3 genome under replicate cell cultures and after application of a thermal stress.

In this work, long-read sequencing was applied for the first time on a Taiwanese isolate of CyHV-3, which had been passaged 100 times on cell culture. Genome analyses revealed that SVs were the major source of polymorphism. SVs displayed a wide distribution along CyHV-3 genome and exhibited high turnover dynamics, with a clear bias towards inversion and deletion events. Analysis of the ORF150 region in ten intermediate cell passages highlighted several deletions, inversions and insertions that deeply altered the structure of ORF150. Results suggested that SV turnovers and defective genomes represent key drivers in the viral population dynamics and *in vitro* evolution of CyHV-3.

For the *in vivo* study, 236 fish from 43 different carp farms of Indonesia were screened for the presence of CyHV-3. Results confirmed a high prevalence of CyHV-3 (nearly 70%), both

in symptomatic and asymptomatic common carp. However, in contrast with the results obtained *in vitro*, long read sequencing of the ORF150 region revealed a low level of genetic diversity and the absence of the 1363-pb deletion.

To address the third objective, an Italian isolate was propagated in 10 identical subcultures and serially passaged 50 times under identical experimental conditions. A thermal stress was applied at passage 26 (P26). Analysis of genome-wide sequences revealed that SNPs accumulated steadily after successive passages, with a uniform distribution along the genome. The thermal stress had apparently no effect on their accumulation in the viral population. In contrast, the accrual of SVs was slower and was obviously affected by the thermal stress. Interestingly, CyHV-3 showed stronger and earlier cytopathic effects from P38 onwards. This observation was concomitant with the fixation in the viral genomes of all subcultures of a specific SNP in ORF131, which was previously demonstrated to be involved in an increased *in vitro* fitness of CyHV-3. Finally, no deletion in ORF150 could be detected, contrary to what could be expected from the first study.

The results presented here suggest that SVs play a substantial role in the evolution of CyHV-3 at least *in vitro*, when environmental pressures are absent. They indicate that ORF150-del haplotype appeared randomly and highlight how fundamental research in virology may answer applied questions.

Key-words : *Cyprinid herpesvirus 3*, evolution, structural variations, cell culture, long-read sequencing, Indonesia

Résumé

L'évolution des virus résulte d'un compromis entre diversification génétique et sélection naturelle. Bien que les mutations ponctuelles (SNP) aient longtemps été considérées comme les principaux moteurs de diversité génétique, il est désormais admis que les variations structurales (VS) peuvent jouer un rôle important dans la diversité des virus. Le *Cyprinid herpesvirus 3* (CyHV-3) est l'un des virus aquatiques les plus pathogènes. Il infecte principalement la carpe commune et la carpe koi, et provoque des mortalités massives pouvant atteindre 100 %. Le CyHV-3 existe dans son hôte sous la forme d'une population de variants génétiquement proches, ou haplotypes.

Une étude antérieure a révélé que la composition des assemblages d'haplotypes peut rapidement varier au cours de passages en série réalisés *in vitro*, entraînant des changements phénotypiques rapides. En particulier, une délétion de 1363 pb dans l'ORF150 (ORF150-del) a été associée à une perte de virulence. Ces résultats ont posé les bases du présent travail, qui s'articule autour de trois objectifs principaux : 1) mieux comprendre les mécanismes évolutifs qui sous-tendent ces assemblages d'haplotypes en culture cellulaire, avec un intérêt particulier pour l'ORF150 ; 2) étudier la prévalence de la délétion ORF150-del *in vivo* ; 3) explorer les dynamiques d'accumulation des VS et des SNP dans le génome du CyHV-3 dans des répliques de cultures cellulaires et après l'application d'un stress thermique.

Un séquençage longs fragments a été réalisé pour la première fois sur un isolat taïwanais du CyHV-3, préalablement passé 100 fois sur culture cellulaire. Les résultats de séquençage ont révélé que les VS étaient la principale source de polymorphisme. Les VS étaient distribuées uniformément sur le génome viral et présentaient une dynamique de renouvellement élevée. L'analyse de la région ORF150 au cours de dix passages intermédiaires a mis en évidence plusieurs délétions, inversions et insertions. Les résultats suggèrent que les VS et la présence de génomes défectifs représentent des facteurs clés dans l'évolution *in vitro* du CyHV-3.

Pour l'étude *in vivo*, 236 poissons provenant de 43 élevages de carpes en Indonésie ont été examinés pour détecter la présence du CyHV-3. Les résultats ont confirmé une forte prévalence du CyHV-3 (de près de 70 %), tant chez les poissons symptomatiques

qu'asymptomatiques. Cependant, contrairement aux résultats obtenus *in vitro*, le séquençage longs fragments de la région ORF150 a révélé un faible niveau de diversité génétique et l'absence de la délétion de 1363 pb.

Pour répondre au troisième objectif, un isolat italien de CyHV-3 a été amplifié en parallèle dans 10 sous-cultures identiques, puis passé 50 fois en série dans des conditions expérimentales identiques. Un stress thermique a été appliqué au passage 26 (P26). L'analyse des séquences génomiques a révélé que les SNP s'accumulent de façon régulière au cours des passages successifs, avec une distribution uniforme le long du génome. Le stress thermique n'a apparemment pas eu d'effet sur leur accumulation dans la population virale. En revanche, l'accumulation des VS était plus lente et manifestement affectée par le stress thermique. Les effets cytopathiques liés à l'infection se sont révélés plus sévères et plus précoces à partir du 38^{ème} passage. Cette observation a coïncidé avec l'apparition d'un SNP spécifique dans l'ORF131, dont une étude récente a montré qu'il était impliqué dans l'augmentation de la virulence du CyHV-3 *in vitro*. Enfin, aucune délétion dans l'ORF150 n'a pu être détectée, contrairement à ce que l'on pouvait attendre de la première étude.

Les résultats présentés ici suggèrent que les VS jouent un rôle majeur dans l'évolution du CyHV-3 au moins *in vitro*, en l'absence de pressions environnementales. Ils indiquent que l'haplotype ORF150-del est apparu de manière aléatoire et mettent en avant l'intérêt de la recherche fondamentale en virologie pour répondre à des questions appliquées.

Mots-clés : *Cyprinid herpesvirus 3*, évolution, variations structurales, culture cellulaire, séquençage longs fragments, Indonésie

List of abbreviations

DNA	Deoxyribonucleic acid
AngHV-1	Anguillid herpesvirus
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
CCB	Common carp brain
CEV	Carp edema virus
CNGV	Carp interstitial nephritis and gill necrosis virus
CPE	Cytopathic effect
CyHV-1	Cyprinid herpesvirus 1
CyHV-2	Cyprinid herpesvirus 2
CyHV-3	Cyprinid herpesvirus 3
dpi	Days post-infection
DVG	Defective viral genome
E	Early (gene)
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharides
EU	European union
FAO	Food and Agriculture Organization
FHM	Fathead minnow
FISH	Fluorescence in situ hybridization
FL	Francois Lieffrig
HCMV	Human cytomegalovirus
HSV	Herpes simplex virus
HTS	High throughput sequencing
IcHV-1	Ictalurid herpesvirus 1
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early (gene)
IHNV	Infectious hematopoietic necrosis virus
IL-10	Interleukin 10

KCF-1	Koi caudal fin 1
KF	Koi fin
KHV	Koi herpesvirus
KHVD	Koi herpesvirus disease
KHV-I	Cyprinid herpesvirus 3 Israel
KHV-J	Cyprinid herpesvirus 3 Japan
KHV-T	Cyprinid herpesvirus 3 Taiwan
KHV-U	Cyprinid herpesvirus 3 United States
kbp	Kilobase pairs
L	Late (gene)
LAB	Lactic acid bacteria
MAS	Motile aeromonad septicemia
MEM	Minimum essential medium
MOI	Multiplicity of infection
NGS	Next-generation sequencing
OIE	Office international des epizooties
ORF	Open reading frame
PCR	Polymerase chain reaction
pi	post infection
qPCR	Real-time quantitative polymerase chain reaction
RaHV-1	Ranid herpesvirus 1
RING	Really interesting gene
RT-PCR	Reverse transcriptase polymerase chain reaction
SAV	Salmon alphaviruses
SNP	Single nucleotide polymorphism
SV	Structural variation
SVCV	Spring viremia of carp virus
SOFIA	State of World Fisheries and Aquaculture
TEM	Transmission electron microscopy
TK	Thymidine kinase
TNFR	Tumor necrosis factor receptor

TR	Terminal repeat
UK	United kingdom
UTR	Untranslated region
US	United states
VNN	Viral nervous necrosis
VNTR	Variable number tandem repeats
VTP	Virion transmembrane protein
WAHIS	World animal health information system
WGS	Whole-genome sequencing
WOAH	World organisation for animal health

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1. Introduction

1.1 Current state of Aquaculture

Aquaculture is a primary global industry contributing to food security and has experienced rapid development in recent years (Belton et al., 2018 ; FAO, 2022). The contribution of aquaculture to global fish production reached 49.0% in 2020 (FAO, 2022; **Figure 1**). From 1990-2020, total world aquaculture expanded by 609%, with an average growth rate of 6.7% per year. The total production from both inland and marine aquaculture comprises aquatic animals (87.5 million tons) and algae (35.1 million tons) (**Figure 2**). Aquatic animals are primarily used as human food. Major farmed aquatic animals consist of finfish (57.5 million tons), molluscs (17.7 million tons) and crustaceans (11.2 million tons). The finfish production remains steady and accounts for around 66% of this production (FAO, 2022). Fish farming has been dominated by Asia for the last 20 years, which produced more than 90% of aquatic animals in the global total volume in 2020. China (49.6 million tons) has produced more farmed aquatic animals than the rest of the world, followed by India, Indonesia, Vietnam, and Bangladesh (**Figure 3**).

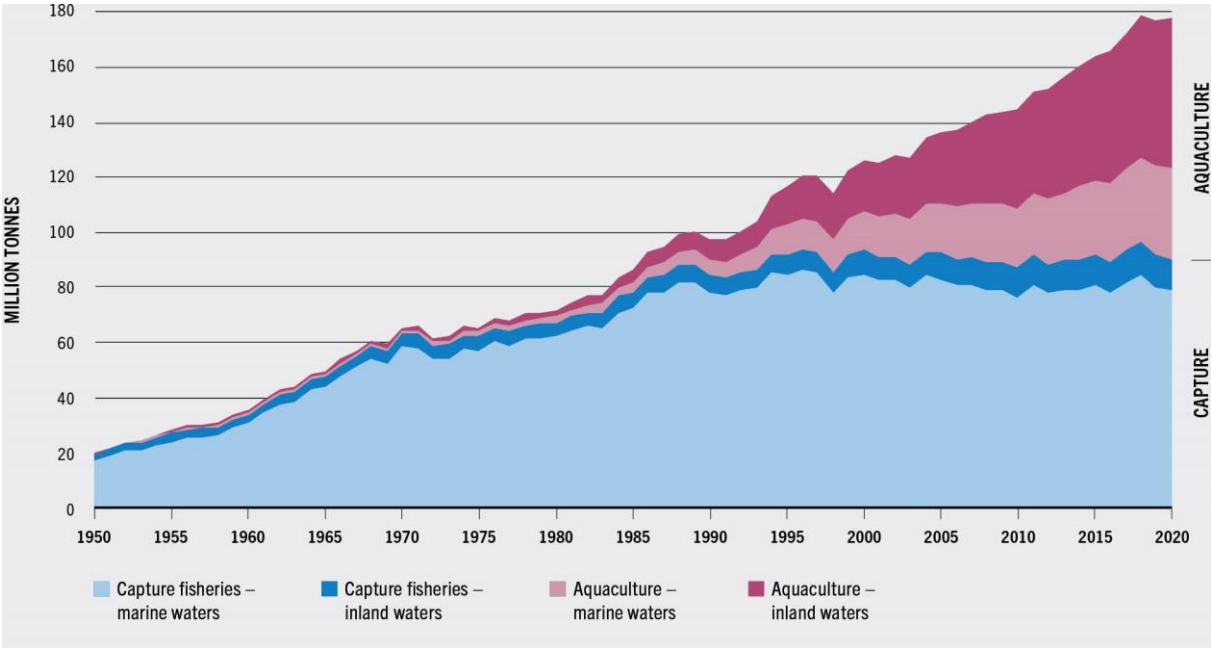


Figure 1 : Global production of capture fisheries and aquaculture production, excluding aquatic mammals, crocodiles, alligators, caimans and algae. Data presented in live weight equivalent (FAO, 2022)

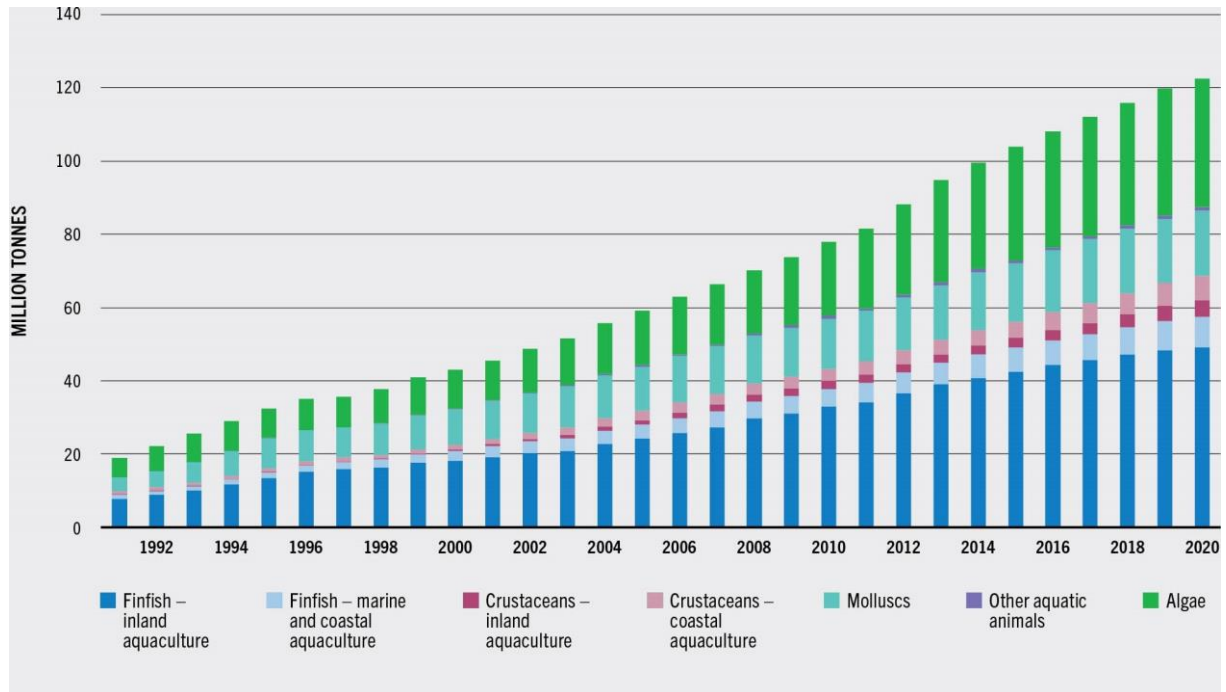


Figure 2 : World aquaculture production from both inland and marine sectors (1991 to 2020) (FAO 2020)

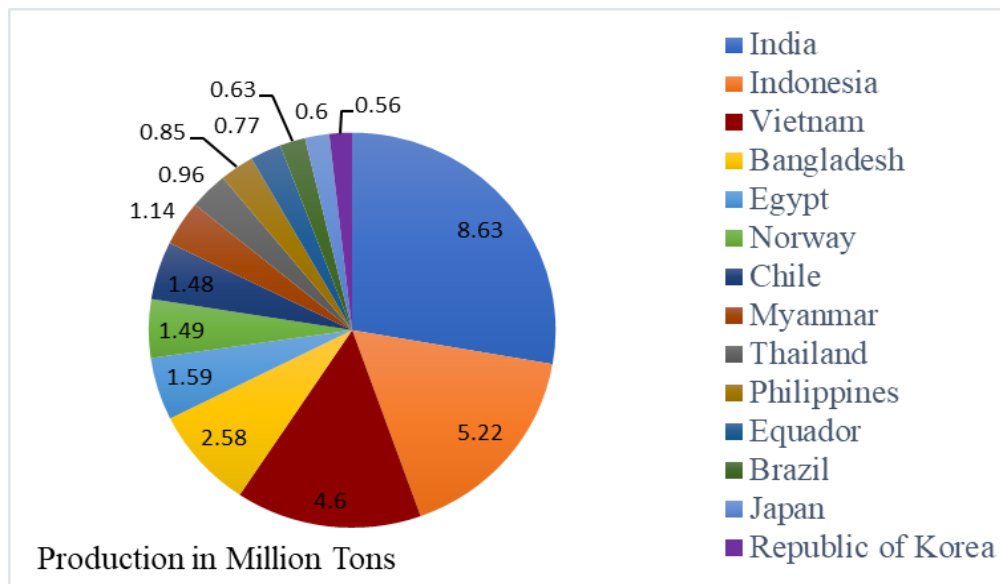


Figure 3 : Major animal aquaculture producing countries, excluding China (FAO 2022)

Fish and seafood are essential to the human diet, providing 3.1 billion people with almost 20% of their daily animal protein intake (FAO, 2016). According to the FAO (2022), providing the needs of an expanding global population with rising living standards is a challenge. It is estimated that by 2030 and 2050, there will be an increase in the demand for food and feed supply of 30% and 70%, respectively (Bruijn et al., 2018). In 2030, about 59 percent of the aquatic food available for human consumption is expected to originate from aquaculture production (SOFIA, 2022). The continuous expansion of aquaculture production may help to relieve the capture fisheries from inland and offshore sources. This increase in production can mainly be attributed to the progress of Asian nations through aquaculture yields (**Figure 2**). Conversely, the decline in marine fishing is attributed to the issue of overfishing. Hence, aquaculture is expected to overtake total capture production. This sector therefore plays a crucial role in food production, food security and income and job sources (Adam and Gunn, 2017).

Aquaculture continues to develop worldwide and is expected to play an important role in the socio-economic development of Global South countries (FAO, 2022). However, increasing aquaculture sustainability in the future requires a combination of practices, policies and innovations that ensure environmental, social and economic balance. Applying good management practices is mandatory to optimise production and limit negative side effects; for instance by minimising the introduction of non-native species that can disrupt local ecosystems (Diana, 2009). Additionally, aquaculture optimisation will only be achieved by involving stakeholders and by developing mechanisation of fish farm operations such as the utilisation of automated feeding systems (Bartley et al., 2006). Strengthening regulations and enforcement mechanisms is also required to prevent illegal practices, overfishing and environmental degradation. Fish farmers need to adopt good practices to avoid environmental and welfare problems. For example, ecosystem-based approaches considering ecological interactions within the aquatic environment (such as integrated multitrophic aquaculture) should now be applied at a larger scale. Responsible aquaculture has a great role to play in enhancing livelihoods and making positive contributions to environmental well-being (Verdegem et al., 2023).

The State of World Fisheries and Aquaculture (SOFIA) reported that in 2030, the production of freshwater finfish will constitute around 60% of the global output in aquaculture. Cyprinids hold the predominant share of global production within the realm of fish and are

recognized as the most economically significant family (FAO, 2022). Of the top five aquaculture species, four are classified under the *Cyprinidae* family: grass carp *Ctenopharyngodon idella* (5.7 million tons, 11.8%), silver carp *Hypophthalmichthys molitrix* (4.8 million tons, 10%), common carp *Cyprinus carpio* (4.2 million tons, 8.6%) and bighead carp *Hypophthalmichthys nobilis* (3.1 million tons, 6.5%). The species within this family hold considerable significance, not only asserving as a valuable food source, but also in the ornamental sector. The Czech Republic, Poland, Hungary, Germany and Croatia are major carp-producing nations within the European Union.

High demand for aquatic food has initiated the expansion of aquaculture production and the commercialization of aquatic products. Both intensification and diversification of aquaculture products are carried out to meet consumer needs. However, intensive aquaculture activities cause environmental and health problems due to the inappropriate use of chemical components and drugs (Reverter et al., 2014). These components are sources of environmental pollution resulting from aquaculture production activities. Adverse chemical products such as hormones, antibiotics, anti-parasites antifungals, anti-inflammatory drugs and anaesthetics, often end up in the environment. Commercial feed has also the potential to introduce pollutants into the pond environment because manufacturers still include antimicrobial substances (Thornber et al., 2022). Environmental impact of aquaculture wastes before and after production can affect non-target organisms, including plants, zooplankton and living microorganisms (Orobets et al., 2019). Significant effects of many antibiotics and hormones on biodiversity have been extensively reported.

Expanding aquaculture industry has attracted considerable controversy including social and ecological impacts of aquaculture (positive and negative) (Young et al., 2019 ; Nielsen et al., 2023). Therefore, it is necessary to increase ecological intensification, i.e. raise production levels by promoting aquatic health management. Naylor et al., (2021) state that aquaculture has become more effectively incorporated into the worldwide food system.

1.2 Infectious diseases of aquatic animals

Global intensification of aquaculture and expanding the number of cultivated species has led to a multiplication of disease outbreaks (Naylor et al., 2021). Semi-intensive and intensive systems have increased the density and presence of post-production waste

(Henriksson et al., 2018 ; Edwards, 2015). Consequently, the application of intensive cultivation systems faces several issues, such as decreased aquatic environment quality, decreased fish growth, increased level of fish stress and increased virulence of infectious agents (Joffre et al., 2018). In the extensive aquaculture systems, infectious diseases are commonly caused by endemic pathogens, parasites and bacteria. Subasinghe, (2005) pointed out that movements of live aquatic animals and animal products (broodstock, seed, and feed) have accelerated the accidental spread and incursion of diseases into new populations and geographic regions, for example, through movements of hatchery-produced stocks.

Infectious diseases are considered as one of the main constraints for aquaculture production, and are caused by a wide array of pathogens including viruses, bacteria, fungi and parasites (Stentiford et al., 2012, 2017). The characteristics of pathogens influence the degree of pathogenicity. Many pathogens directly impact the outcome of their interactions with the host and the environment. Some pathogens are necessarily dependent on a host to complete their life cycle (obligate pathogens), while others can persist/survive without the presence of a host (facultative pathogens).

The typical fish pathogenic bacterial species that hamper fish cultivation belong to the genera *Vibrio*, *Aeromonas*, *Flavobacterium*, *Yersinia*, *Edwardsiella*, *Streptococcus*, *Lactococcus*, *Renibacterium* and *Mycobacterium* (Bruijn et al., 2018 ; Buján et al., 2018 ; Mugetti et al., 2020 ; Viel et al., 202 ; Irshath et al., 2023). Vibriosis, a significant bacterial disease impacting fish, bivalves and crustaceans, is primarily attributed to pathogenic species like *Vibrio anguillarum* and *V. harveyi* (Ina-Salwany et al., 2019 ; Sanches-Fernandes et al., 2022) . In the *Flavobacterium* genus, which consists of more than 30 species, *Flavobacterium psychrophilum*, *F. branchiophilum*, and *F. columnare* stand out as critical disease-causing agents affecting salmonids, catfish, and other cultured species (Starliper, 2011 ; LaFrentz et al., 2022).

There are various important parasitic groups for aquaculture: they include oomycetes (genus *Saprolegnia*), protozoans (amoebae, flagellates, ciliates) and metazoans (myxozoans, monogeneans, digeneans, cestodes, nematodes, crustaceans) (Buchmann, 2022). The typical external parasite infections encompass *Trichodina spp.*, *Gyrodactylus spp.*, *Trichodinella spp.*, *Ambiphrya spp.*, *Apiosoma spp.* and *Epistylis spp.* (Blazhekovikj-Dimovska and Stojanovski, 2021). Other significant protozoan parasites may affect aquaculture, including the

Ichthyophthirius, *Ichthyobodo* and *Myxobolus*. In their natural environment, these parasites exist in small quantities on the fish and generally do not pose a threat. Nevertheless, specific circumstances such as inadequate water quality may substantially increase their population, resulting in significant risk for the host (Hutson et al., 2022).

Viruses are obligate intracellular parasites that pose a crucial threat to aquaculture production (Sahoo and Goodwin, 2012). Viral diseases have caused enormous economic losses to aquaculture and have also affected valuable stocks of wild aquatic animals (Kibenge, 2019 ; Mugimba et al., 2021).

The five primary diseases reported in aquaculture species are *Ichthyophthirius multifiliis* parasitic disease in freshwater fish, motile aeromonad septicaemia (MAS) disease caused by *Aeromonas hydrophila* bacterial infection, Koi herpesvirus disease (KHVD) in carp and koi caused by Koi herpesvirus (KHV, also named *Cyprinid herpesvirus 3* (CyHV-3), white spot syndrome virus (WSSV) disease in penaeid shrimp and viral nervous necrosis (VNN) disease in grouper (Walker and Winton, 2010). Infectious diseases cause tremendous economic losses, globally estimated between US\$ 1 to US\$ 10 billion/year (Shinn et al., 2015). Concerns about unfavourable consequences of hatchery-reared seed releases, such as the spread of pathogens, competition for food, and cannibalism, as well as genetic deterioration of wild stocks, have been increasing within the framework of aquatic animal health management (Fushimi, 2001).

Virulence of microorganisms is influenced by changes in the aquatic ecosystem that favour their spread and growth. Other stressors, such as water quality, fish density or unbalanced nutrition, are also known to facilitate fish pathogens to become more virulent (Leung and Bates, 2013). High-density rearing of aquatic animals and warmer temperatures provide ideal conditions for outbreaks that cause high mortalities and rapid progression of diseases (Salama and Murray, 2011). Physiological stresses and immune system deficiencies of aquatic animals increase their susceptibility to pathogen exposure and, in turn, the transmission of diseases (Salama and Murray, 2011). For instance, opportunistic agents may not usually be harmful, but they can become pathogenic and cause infections when aquatic organisms are stressed and have compromised immune systems. Thus, special efforts are required to prevent disease spread due to the release of cultured seeds into the ecosystems regarding aquatic animal health management (Brian, 2023).

1.3 Disease prevention and curative efforts

Control of aquatic pathogens primarily relies on the rapid detection of infected sites to prevent further spread. Prevention through proper biosecurity measures includes quarantine procedures and health monitoring. Efforts have been made on prophylaxis and treatment measures, and enhancement of animal health through nutrition (Immanuel et al., 2012 ; Barman and Nen, 2013 ; Nafiqoh et al., 2020). Furthermore, the use of probiotics, prebiotics and synbiotics could also play a role in preventing and controlling outbreaks. Another way to prevent infectious diseases in aquaculture is implementation of selective breeding programs to develop fish strains that are more disease-resistant (Dixon et al., 2009 ; Palaiokostas et al., 2019 ; Hwang et al., 2020).

Controlling opportunistic pathogenic bacteria in aquaculture is commonly conducted through prophylactic chemotherapy or antibiotics (Cabello et al., 2013 ; Miranda et al., 2018). The imprudent and misguided application of antimicrobial agents to combat diseases is adverse to the progress and sustainability of the aquaculture industry. Disease control methods in mass aquaculture production frequently involve antibiotics, especially in countries where regulatory limits may not be well monitored (Santos and Ramos, 2016). In some countries, the utilisation of antibiotics has been prohibited due to its significant risks of stimulating the proliferation of antibiotic-resistant bacteria and resulting in antibiotic residues within farmed organisms and environmental pollution (Watts et al., 2017 ; Adenaya et al., 2023). Antibiotic-resistant bacteria and antibiotic residues within fishes may then pose a safety concern for human consumption due to the potential for transmission to humans (Sargenti et al., 2020). Parasitic diseases are typically treated using chemical interventions (Norbury et al., 2022).

Antibiotics cannot control viral diseases. When aquatic animals are infected with viral diseases, the use of antibiotics by unprofessional farmers not only fails to alleviate the condition but also exacerbates water pollution, resulting in an increase in mortality instead of a decrease (Liao et al., 2022b). Some studies showed that resistance breeding and crossbreeding may mitigate viral infections (Mugimba et al., 2021). Immunostimulating substances have also been tested to prevent virus replication (Immanuel et al., 2012 ; Kang et al., 2012). In addition, probiotics have promising potential to reduce the incidence of viral diseases in aquaculture (Lakshmi et al., 2013 ; Harikrishnan et al., 2010). In numerous cases, vaccination plans are more promising to combat viral diseases. Possible solutions for controlling viral diseases are

using live attenuated vaccines, inactivated vaccines or most specific vaccines like DNA vaccines, subunit vaccines, and vaccines with recombinant viral proteins (Su and Su, 2018 ; Du et al., 2022 ; Kembou-Ringert et al., 2023). Viral attenuation is generally a consequence of mutations that are introduced during viral replication and accumulate during serial passages in cell cultures. Consequently, the reversion to a pathogenic phenotype cannot be excluded and needs to be carefully checked. Some commercialised vaccines are available for global use, including inactivated vaccines of AQUAVACÒ against *Iridiovirus*, PD NorvaxÒ Compact PD against Salmon alphaviruses (SAV) (Dhar et al., 2014), and inactivated viral vaccine against Infectious hematopoietic necrosis virus (IHNV).

A more sustainable approach to disease control, safeguarding fish and shellfish against resurgent diseases, could be implemented by using prophylactic agents. Most metabolites of viable lactic acid bacteria exhibited antiviral properties in shrimp and fish cultures (Vilhelmova-Ilieva et al., 2023). However, it is difficult to eliminate a virus from contaminated water once the infection is established. Disinfecting methods are also frequently applied in aquaculture, as for example draining, UV-irradiation, ozonation, heating and incubation with quicklime (Kasai et al., 2002 ; Teitge et al., 2020). Moreover, some studies highlight the limitations of conventional disinfectants in aquaculture, leading to restricted usage.

Several significant constraints of disease control in aquaculture are limited quality control and wastewater management regulations and a need for coordination between government bodies to design the best aquaculture production framework (Assefa and Abunna, 2018 ; Joffre et al., 2018).

1.4 Common carp production and associated diseases

1.4.1 Carp production

Common carp *Cyprinus carpio* Linnaeus, 1758, is a freshwater fish native to Eurasia and introduced to every part of the world. Common carp is the oldest domesticated fish (Balon 1995 ; Nedoluzhko et al., 2020). This species is a member of the *Cyprinidae* family, the most prominent family in freshwater fish. Common carp is one of the main cultivated fish, not only for human consumption, but also for ornament, since the colourful koi is highly sought-after (**Figure 4**). The natural distribution of common carp and the domestication process have led to the emergence of several carp varieties, suggesting a wide range of genetic resources (Yuan et

al., 2018). Koi is selectively bred as a valuable, visually stunning, and colourful ornamental fish for competitive displays and exhibitions. Koi has significant economic, recreational, and cultural value in many parts of the world (Rahman, 2015).

Wild common carp inhabit rivers, lakes, large and small artificial and natural reservoirs in slow or fast-moving water systems. However, they prefer lower-moving water bodies with soft sediments. Common carp have a wide tolerance to many environmental and food contexts (Adámek et al., 2019). They are tolerant to many environmental factors such as temperature (2 - 40°C), salinity (up to about 14 parts per thousand), pH (from 5 to 10) and oxygen (Takahara et al., 2014 ; Homoki et al., 2021). Common carp are omnivorous freshwater fish that consume a wide range of food items, including detritus, insects, macrophytes, phytoplankton, ostracods, zooplankton and gastropods (Saikia and Das, 2009 ; Horvath et al., 2023). **Figure 5** illustrates an overview of the distinctive developmental stages of common carp. The economically viable individual weights for various age categories of common carp are approximately 0.025-0.05 kg per fingerling and 0.25-0.5 kg per grower. The size range for table fish of common carp can vary depending on the country, between 0.25 and 3 kg (**Table 1**). Carp also have a high reproductive capacity and may thrive in various aquatic habitats (Woynarovich, 2011). These are the reasons why this fish is widely distributed and found in most types of freshwater habitats (Rahman et al., 2018).

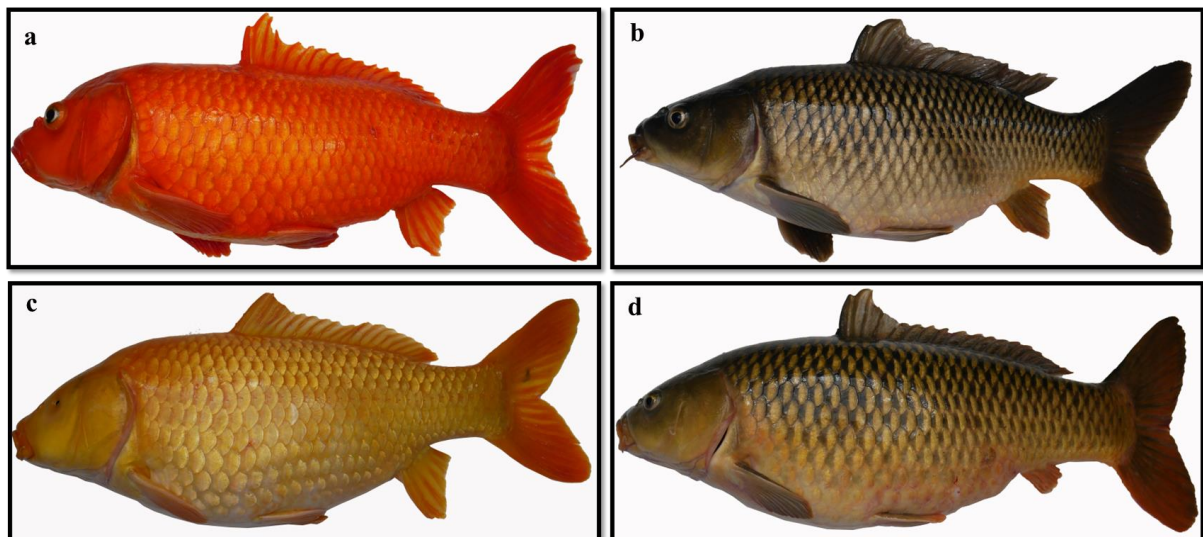


Figure 4 : Several strains of Common carp from Indonesia. a) Strain Cangkringan; b) Strain Mustika; c) Strain Sinyonya; d) Strain Punten. Pictures were kindly provided by Ucu Cahyadi from main Center for Freshwater Aquaculture Development, Sukabumi, Indonesia

Table 1 : Summary of water quality requirement and duration for different life stages of common carp (*Cyprinus carpio*) in pond fish-farming system (FAO, 2023).

	Rearing			
Life stage	Climate	Duration	Description	Final weight (g)
Larva	Under all climates (water temperature ≥20 °C)	3–7 days	Mixed feeding and the first days of only internal feeding	0.025–0.05
Fry		15–30 days	From feeding larva to advanced fry	0.2–1.0
Fingerling		45–85 days	From fry to fingerling	25–50
Grower		120–170 days	From fingerling to grower	250–500
Table fish		120–170 days	From grower to table fish	250–500
Brood fish	Temperate	2–4 years	From table fish to brood fish	>4 000
	Subtropical	1–2 years		>3 000
	Tropical	0.75–1 year		>2 000

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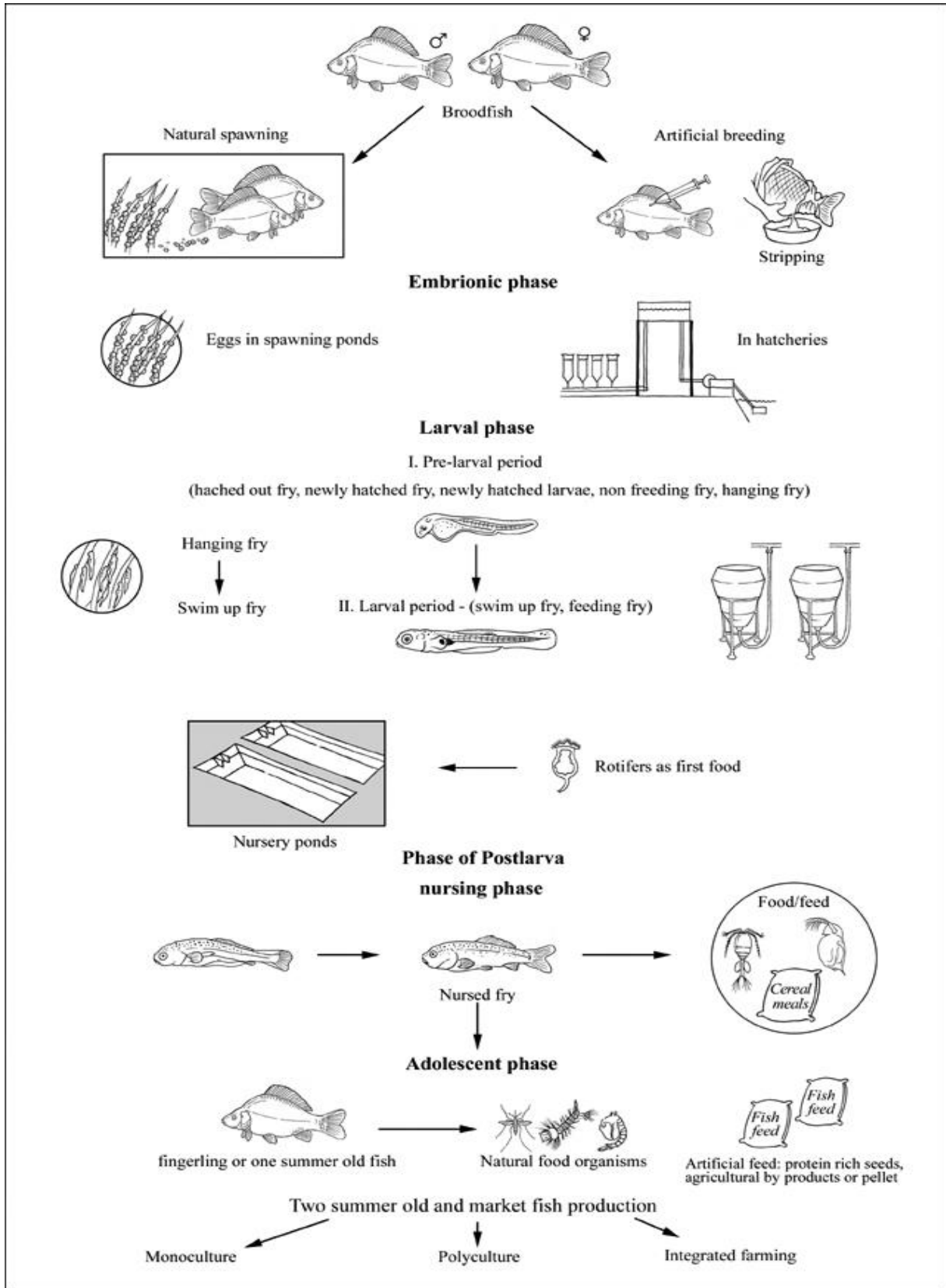


Figure 5 : Production cycle of *Cyprinus carpio* (FAO, 2022).

The major carp production countries are located in Asia, particularly China and Indonesia, where production amounted to 86.2% of the global production in 2015 (FAO, 2020). In Central and Eastern Europe, carp is the most cultured fish, accounting for more than 80% of total fish production (Adamek et al., 2012 ; Anton-Pardo et al., 2014). The rearing systems of the common carp are highly diversified: extensive monoculture in earthen ponds, with or without fertilisation and additional feeding; intensive monoculture in small ponds, cages, or raceways; and integrated mono- or polyculture with other agricultural activities. The most widely used farming systems consist of ponds (earthen ponds or running water ponds) and floating net cages (**Figure 6**). Cultured ornamental carp using an aquarium or pond in a recirculating system has better outcomes in terms of production, performance and cost (Henrique et al., 2019).



Figure 6 : Farming systems of carp aquaculture. Upper panel: floating net cages in the lake Toba, North Sumatra. Lower panel: floating net cages and ponds in an artificial reservoir (Personal documentation).

1.4.2 Carp viral diseases

Viral diseases are a significant threat to the production and quality of common carp. Aquatic viruses have serious ecological impacts when transmitted into natural habitat carriers, enabling the infection of naive fish (Ito et al., 2013). The introduction route of viral diseases in cyprinids can be facilitated through the international trade of live fish (Jeoung et al., 2023). There are several viruses that affect carp production, including the families *Rhabdoviridae* (Spring viraemia of carp virus, SVCV), *Poxviridae* (Carp oedema virus, CEV) and *Alloherpesviridae* (Cyprinid herpesvirus 1, CyHV-1; Cyprinid herpesvirus 2, CyHV-2; Cyprinid herpesvirus 3, CyHV-3) (Badhusha et al., 2022 ; Ziafati Kafi et al., 2022). SVCV induces an acute haemorrhagic viral infection in carp, particularly common carp (Ahne et al., 2002). CEV disease, also known as sleepy koi disease, is caused by a poxvirus associated with outbreaks in koi and common carp (Zhang et al., 2017 ; Jin et al., 2020 ; Adamek et al., 2021). Cyprinid herpesviruses are restricted in their host range: CyHV-1 and CyHV-3 are only known to cause disease in common carp strains, and CyHV-2 is only known to cause disease in goldfish (*Carassius auratus*) and in the closely related Prussian carp (*Carassius gibelio*). Recently, cases of mass mortality caused by co-infections of CyHV-3/CEV have been reported worldwide (Adamek et al., 2019 ; Kim et al., 2020 ; Joung et al., 2023). In addition, Ito et al. (2013) indicated that CyHV-2 is transmitted into natural habitat carriers and can infect naive fishes in low water temperature conditions.

1.5 Koi herpesvirus disease and *Cyprinid herpesvirus 3*

1.5.1 Taxonomy of herpesviruses

Herpesviruses are widely distributed in nature, well-adapted to their natural host, and can be found in many different species. The order *Herpesvirales*, whose ICTV (International Committee on Taxonomy of Viruses) classification was revised in 2022, is divided into three related families infecting various animals: *Orthoherpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae* (Pellett et al., 2012 ; ICTV 2022). Members of this order share similar structural, genetic and biological properties. All members of the order *Herpesvirales* contain a genome consisting of a single and linear dsDNA with a length ranging from 124 to 295 kilobase pairs (kbp) (**Figure 7**). The percentage of guanine plus cytosine (G+C) varies from 31 to 77% (Aoki et al. 2007). To date, four genera (*Batravirus*, *Cyivirus*, *Ictavirus*, *Salmovirus*) and thirteen

species of *Alloherpesviridae* family have been described (ICTV, 2022), with ten infecting fish species (Hanson et al., 2011). Over the last two decades, many studies have been devoted to alloherpesviruses affecting fish (e.g. in genera *Cyvirivirus*, *Ictavirus* and *Salmovirus*; **Table 2**). The genus *Cyvirivirus* contains viruses that infect common carp (Cyprinid herpesviruses, CyHV-1 and CyHV-3), goldfish (Cyprinid herpesvirus 2, CyHV-2) and freshwater eel (Anguillid herpesvirus 1, AngHV-1).

Cyprinid herpesvirus 3 (CyHV-3), renamed *Cyvirivirus cyprinidallo3* in 2022 by the *Herpesvirales* Study Group of the ICTV (ICTV 2022), is the causative agent of the koi herpesvirus disease (KHVD). Its common name is koi herpesvirus (KHV) because it was first isolated from a koi (Hedrick et al., 2000). It was also named carp interstitial nephritis and gill necrosis virus (CNGV), but this name is not used anymore. It induces lethal disease in common carp *Cyprinus Carpio* and the valuable ornamental koi from the same species. For the rest of the manuscript, the names CyHV-3 will be conserved instead of *Cyvirivirus cyprinidallo3*

Table 2 : Alloherpesviruses infecting fish (according to ICTV 2023)

Genus	Species
<i>Cyvirus</i>	<i>Cyvirus anguillidallo1</i>
	<i>Cyvirus cyprinidallo1</i>
	<i>Cyvirus cyprinidallo2</i>
	<i>Cyvirus cyprinidallo3</i>
<i>Ictavirus</i>	<i>Ictavirus acipenseridallo2</i>
	<i>Ictavirus ictaluridallo1</i>
	<i>Ictavirus ictaluridallo2</i>
<i>Salmovirus</i>	<i>Salmovirus salmonidallo1</i>
	<i>Salmovirus salmonidallo2</i>
	<i>Salmovirus salmonidallo3</i>

1.5.2 Emergence of CyHV-3

KHVD was first reported in 1998 after mass mortalities of koi carps in 1996 in both juvenile and adult stages cultivated in Israel, the United States, and European countries (Bretzinger et al., 1999). CyHV-3 was described two years later as the causative agent of KHVD (Hedrick et al., 2000). The first complete genome sequences of CyHV-3 were obtained for three strains isolated from outbreaks in the United States (KHV-U), Israel (KHV-I), and Japan (KHV-J) (Aoki et al., 2007). Sequence comparisons indicated a very high degree of identity (>99.9%) and showed that KHV-U was more closely related to KHV-I than to KHV-J. Since its emergence in 1996, this devastating disease has rapidly spread due to the global fish trade and international ornamental koi exhibitions (Gotesman et al., 2013). CyHV-3 infections are associated with environmental and economic problems, leading to up to 95% mortality (Sunarto et al., 2014 ; Ababneh et al., 2020). In Indonesia, KHVD caused a loss of revenue of approximately US\$25 million in carp production (Sunarto & Cameron, 2006). Yearly carp production decreased by 2.3% in Poland due to KHVD (FAO, 2017). The high virulence of CyHV-3 was highlighted by the World Organization for Animal Health (WOAH, formerly OIE) and CyHV-3 was classified as a notifiable fish disease agent¹. Several factors, such as temperature shift, handling or transportation stress, and concurrent infections, have influenced the host to recurrent disease and virus transmission (Pokorova et al., 2005 ; Fabian et al., 2016 ; Minamoto et al., 2009b; Minamoto et al., 2009a). Weak laws of quarantine and lack of

¹ <https://www.woah.org/en/disease/koi-herpesvirus-disease/>

diagnostic application during the global trade in carp and koi have greatly increased the spread of the disease (Shahin et al., 2020). Hence, the need to protect common and koi carp from the negative impact of CyHV-3 prompted an increasing interest in studying this virus.

1.5.3 Morphological and genomic features of CyHV-3

CyHV-3 is morphologically similar to other herpesviruses and contains many features of their genomic organisation (Waltzek et al., 2005 ; Davison et al., 2013). The CyHV-3 virions comprise an icosahedral capsid of 100-230 nm in diameter containing the dsDNA genome, surrounded by a matrix of proteins called tegument and an envelope with glycoproteins (Miyazaki et al., 2008 ; Cheng et al., 2011; **Figure 8**). With 295 kbp, CyHV-3 has the largest genome among all herpesviruses (Davison et al., 2009). The genome is flanked by two terminal repeats (TR), consisting of identical sequences at both ends of the viral genome. Each TR is approximately 22 kbp in length and plays essential roles in the replication and packaging of the virus genome during its life cycle (Ilouze et al., 2012a). The genome of CyHV-3 contains 156 unique open reading frames (ORFs) predicted to encode proteins (Aoki et al., 2007 ; Michel et al., 2010; **Figure 9**). Although CyHV-3 has a genomic organisation similar to that of other alloherpesviruses, it is exhibited limited homologs, and among the 156 CyHV-3 predicted proteins, only 15 share similarity with CyHV-1 and CyHV-2 proteins, and to a lesser extent with Ictalurid herpesvirus 1 (IcHV-1) and Ranid herpesvirus 1 (RaHV-1) proteins (Aoki et al. 2007 ; Zhang et al., 2023). The least divergent ORFs tend to be conserved among herpesviruses and are involved in essential replication aspects (Ilouze et al., 2012a)

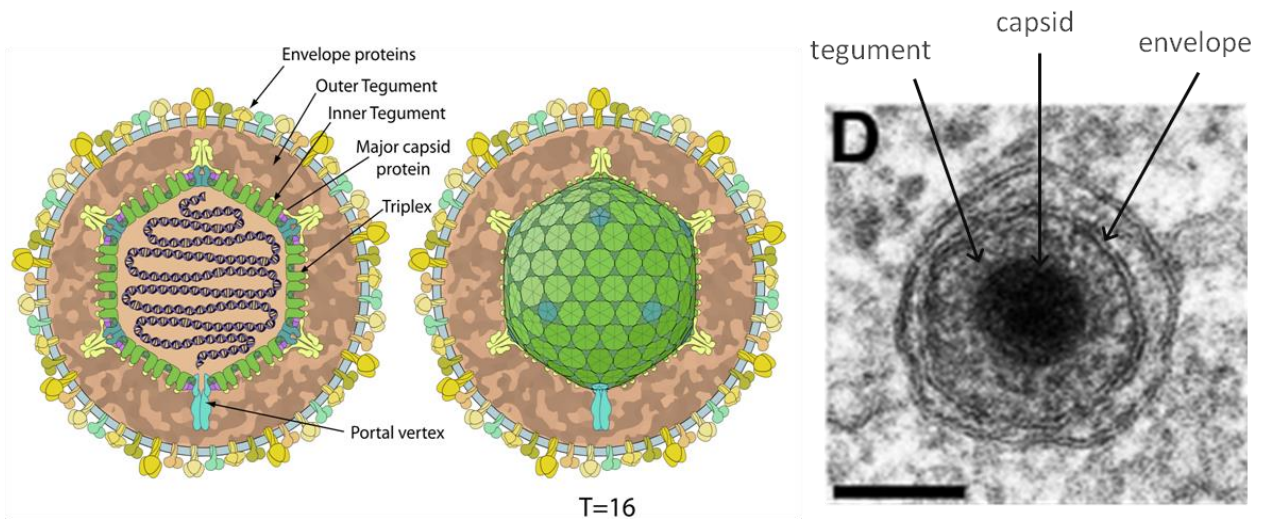


Figure 8 : Family of *Alloherpesviridae* morphology and structure. Left: Family *Alloherpesviridae* structure. Virions are enveloped, spherical to pleomorphic, 150-200 nm in diameter. The capsid consists of 162 capsomers with a T=16 icosahedral symmetry. Glycoprotein complexes are embedded in the lipid envelope ([ViralZone](#); [expasy.org](#)). Right (D): Cyprinid herpesvirus 3 (CyHV-3) morphology. Electron microscopy examination of CyHV-3 virion from CCB cells infected with CyHV-3; virions are enclosed within a cellular vesicle as they are transported towards the plasma membrane. The scale bar represents a distance of 100 nanometers. Adapted from Mand (Mettenleiter et al., 2009).

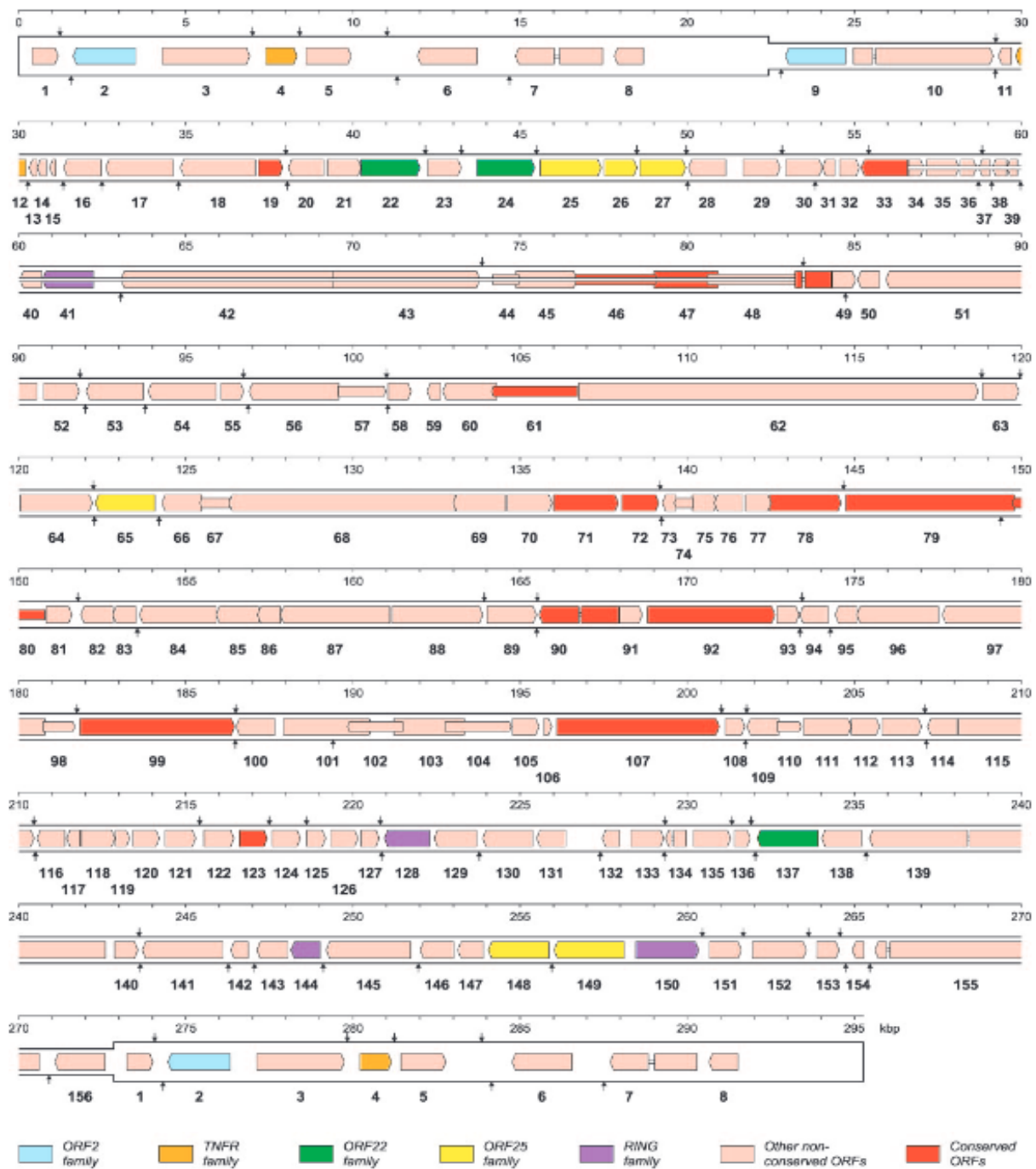


Figure 9 : CyHV-3 genome map. The terminal direct repeats (TR) are shown in the first 8 ORFs and the last 8 ORFs and are marked as a thicker line format than the unique region (U). ORFs predicted to encode functional proteins (based on the strain J coordinates) are distinguished by coloured arrows (the legend at the foot). Colours of protein-coding regions indicate core ORFs that are convincingly conserved among alloherpesviruses, families of related ORFs, and other ORFs (from Aoki et al., 2007).

1.5.4 Proteomic profile of CyHV-3

Information on the putative function of the 156 ORFs is still extremely limited, and so far almost all predictions have been made *in silico* (Aoki et al., 2007). Davison et al. (2013) reported that nine ORFs presented an intron, and eight (ORF1 to ORF8) are duplicated in terminal repeats (**Figure 9**). CyHV-3 genome comprises five distinct gene families: the ORF2 family (ORF2, 3, 9, 129, 130 and 135), the tumour necrosis factor receptor (TNFR) family (ORF4 and ORF12), the ORF22 family (ORF22, 24 and 137), the ORF25 family (ORF25, 26, 27, 65, 148 and 149) and the really interesting new gene (RING) family (ORF41, 128, 144 and 150) (Davison et al., 2013). The ORF25 family consists of six paralogous sequences encoding potential type 1 membrane glycoproteins. CyHV-3 genes are sequentially expressed during infection and divided as follows at permissive temperature : 15 immediate early (IE), 112 early (E), and 22 late (L) genes (Ilouze et al., 2012a)).

A proteomic analysis of purified virus particles identified 40 CyHV-3 structural proteins in mature CyHV-3 virions from a European (FL) isolate (Michel et al., 2010b; **Figure 10**). These proteins were further grouped as three capsid proteins, two tegument proteins, 13 envelope proteins and 22 unclassified proteins. Another proteomic characterisation of extracellular virions from two Chinese (GZ10 and GZ11) isolates and the FL one, highlighted 43 viral proteins (Yi et al., 2014 ; Vancsok et al., 2017). A comparison of the proteomes derived from both studies revealed 39 proteins in common (**Figure 11**). Moreover, four viral proteins detected in Chinese isolates (ORF11, ORF27, ORF91, and ORF116) were absent in the European one (Yi et al., 2014). These interesting studies confirmed, at the protein level, some of the genomic differences reported by Aoki et al. (2007) between European and Asian isolates. Among the viral proteins identified in these studies, pORF92 and pORF62 emerged as the most abundant structural proteins. They were classified as primary capsid and tegument proteins (Yi et al. 2014). Another report showed that pORF12 and pORF134 were the most abundant secreted viral proteins detected by 2-D liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ouyang et al., 2013).

Several other ORFs have been identified in CyHV-3. Three were shown to encode enzymes related to metabolism: a thymidine kinase (ORF55), a dUTPase (ORF123) and a ribonucleotide reductase (ORF141) (Fuchs et al. 2014 ; Rosenkranz et al. 2008). ORF33 was shown to encode a protein involved in DNA packaging. Proteins involved in DNA replication

include DNA helicase (pORF71) and DNA polymerase (pORF79). CyHV-3 genome also encodes proteins potentially involved in immune evasion mechanisms such as G-protein coupled receptor (encoded by ORF16), TNFR homologues (ORF4 and ORF12) and an interleukin-10 (IL-10) homologue (encoded by ORF134) (Aoki et al., 2007). Another study reported that pORF25, pORF92, pORF65, pORF99 and pORF81 can induce a protective immune response (Cui et al., 2015).

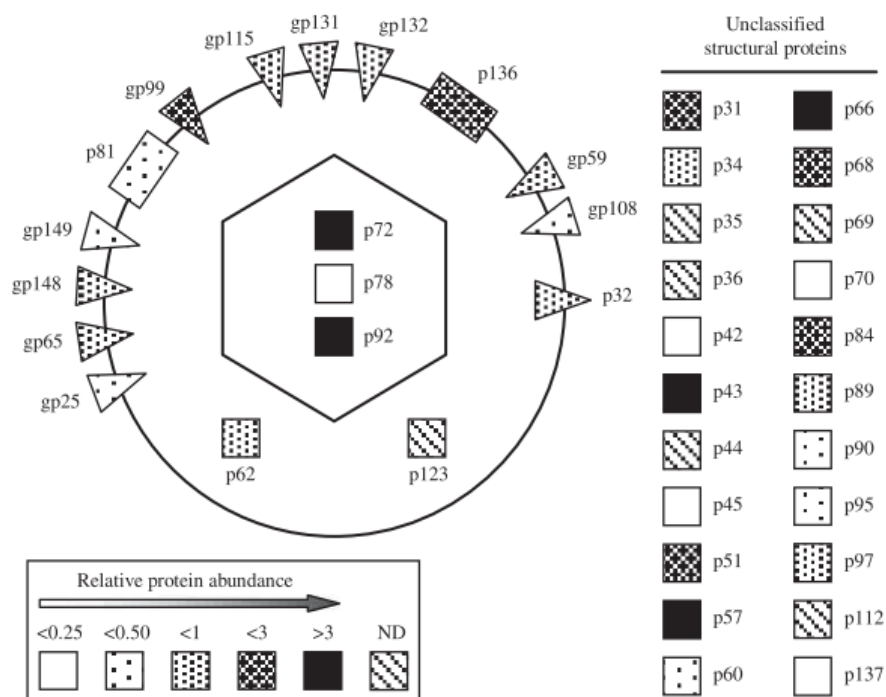


Figure 10 : Proteomic characterisation of extracellular virions detected in FL strain.

The viral particle contains the envelope (circle), capsid (hexagon) and tegument. Membrane proteins are represented of type 1 (triangles pointed inside), 2 (triangles pointed outside) and 3 (rectangles). The different fillings indicate the relative abundance of proteins based on their emPAI (< 0.25, < 0.50, < 1, < 3 and > 3). p: protein, gp: glycoprotein, ND: no data (Michel et al., 2010b).

In 2017, Vancsok et al., highlighted the distinctions in protein-level characteristics between isolates from Europe and Asia and distinguished between essential and non-essential viral transmembrane proteins (VTPs). VTPs play crucial roles in various stages of the herpesvirus infection cycle, such as viral adsorption and entry into the host cell, virion morphogenesis and egress from the host cell. The expression of VTPs on the virion surface may

also make them targets for neutralising antibodies. Using mutagenesis experiments, Vancsok et al., (2017) demonstrated that the FL strain encodes 16 virion transmembrane proteins. Eight of these proteins are essential for viral growth *in vitro*, seven of the nonessential proteins affect viral growth *in vitro*, and two affect virulence *in vivo*. A mutant lacking ORF25 is highly attenuated; it was tested for vaccinating fish but induced moderate immune protection. At least 40 structural proteins have been detected in various CyHV-3 strains, but half remain uncharacterized, among which many VTPs. Research is therefore still needed to identify ORFs and characterise their functions, with the aim of selecting potential candidates for vaccine design.

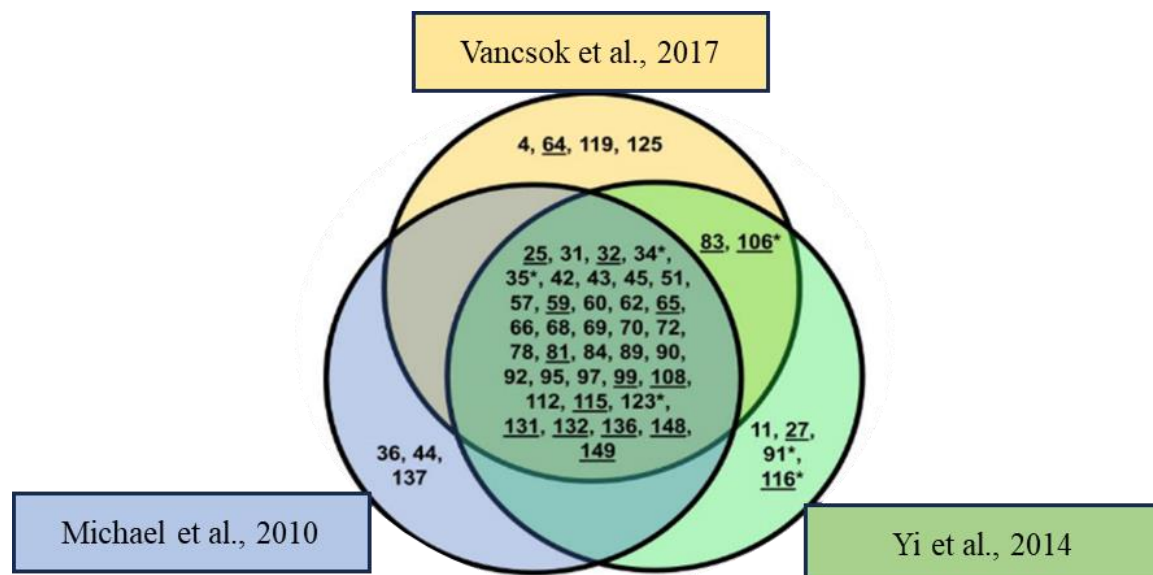


Figure 11 : Proteomic comparison of CyHV-3 virion-associated proteins identified in a European strain (FL) ((Michel et al., 2010b) and two Asian strains (GZ10 and GZ11) (Yi et al., 2014). Green, blue, and orange colours represent the proteins that are detected in publications of Yi et al., (2014), (Michel et al., 2010b) and Vancsok et al., (2017), respectively. Original figure from (Vancsok et al., 2017).

1.6 Epidemiology of CyHV-3

1.6.1 Geographic distribution and prevalence

The initial occurrences of KHVD outbreaks were documented in koi and cultivated carp in Germany (in 1997-1998), England (1996), Israel, and the USA (1998) (Hedrick et al., 2000 ; Haenen et al., 2004). Since then, rapid global dissemination across continents of KHVD and

high mortality were reported. KHVD outbreaks quickly spread to other European nations (**Table 3**), South Africa, Taiwan, China, Thailand, and Japan by the early 2000s (Yuasa et al., 2005 ; Pokorova et al., 2007 ; Pikulkaew et al., 2009 ; Rathore et al., 2012). It is known that the movements of live aquatic animals or their eggs greatly contribute to the dissemination of aquatic viruses. International trade is responsible for the introduction of viruses to new geographical areas, especially since persistent viral infections may occur without clinical signs. This issue has caused enormous economic losses to carp aquaculture (Flegel, 2012 ; Zhang, 2018). The effectiveness of CyHV-3 in infecting the host and rapidly invading new regions is intrinsically linked to its evident capacity to establish persistent infections (Fujioka et al., 2015), its potential to infect a broad host range as carriers (Thresher et al., 2018 ; Torres-Meza et al., 2020a ; Tolo et al., 2023), and its ability to contaminate abiotic vectors (nets, sediments...).

The high number of ornamental carp imported into the UK from non-EU countries, that had potentially been exposed to the virus, is a likely route of virus introduction in Europe (Tolo et al., 2021). Likewise, CyHV-3 spread quickly throughout Indonesia after its first introduction in 2002 (Sunarto and Cameron, 2006; Sunarto et al., 2011). The first documented case of CyHV-3 mortality in Indonesia occurred in March 2002 among koi carp raised in Blitar, East Java (Sunarto et al., 2005). This site had recently introduced fish from China via Hong Kong in January of the same year. The following month, many other outbreak sites occurred among cultured common carp in Subang Regency and floating net cages at Cirata Reservoir, West Java. Subsequently, the disease began to spread to various carp farms nationwide (**Figure 13**) (WAHIS 2023). This outbreak resulted in a devastating 95% mortality rate and financial losses of approximately US\$0.5 million within three months, as detailed by Sunarto et al. in 2005. This is probably what happened at the global scale, and what explains the wide geographic distribution of CyHV-3 in the world (Gathore et al., 2011 ; CABI, 2022; **Figure 12**). Asymptomatic presence is prevalent among carp populations. Besides, exposed fish may be present in some wild systems either as a result of direct stocking, fish escaping from fisheries during flooding, or through exposure to virus release (Taylor et al., 2010 ; McColl et al., 2018 ; Padhi et al., 2019). Carp are also known to live next to the bottom sediments, which aggregate natural and artificial food sources (Hundt et al., 2022). This supports the rapid spread of this virus to various regions. Among 109 rivers in Japan, 93 were demonstrated to be contaminated

with CyHV-3 (Minamoto et al., 2012). Recent information on distribution at the country level is available on the WAHIS (World Animal Health Information System) website².

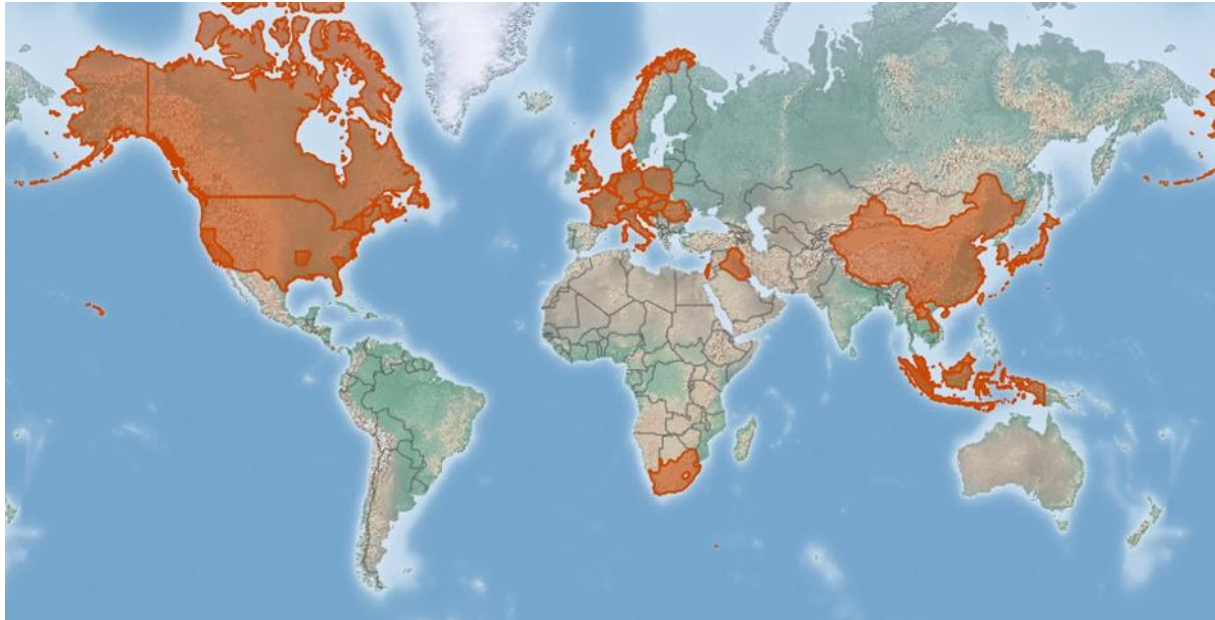


Figure 12 : Distribution map of CyHV-3 (in orange).

Source : <https://www.cabidigitallibrary.org/>

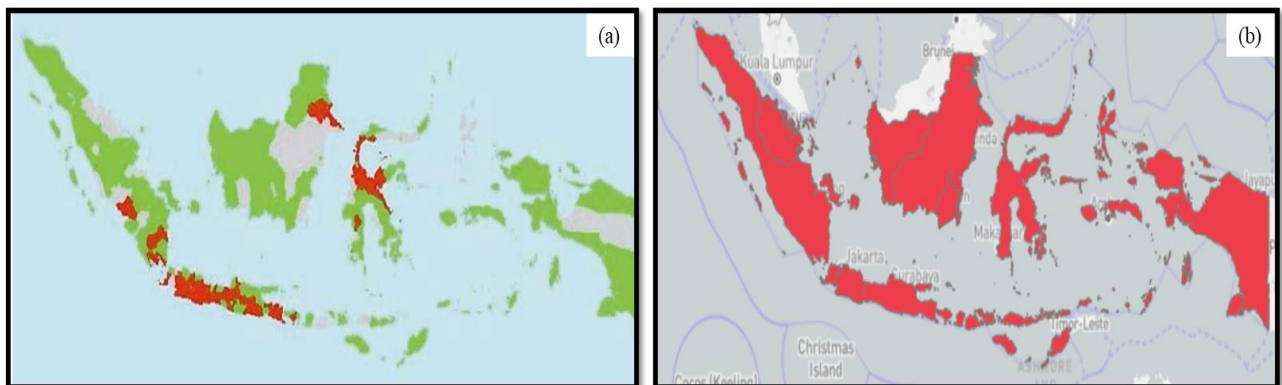


Figure 13 : Geographic distribution of CyHV-3 in Indonesia. CyHV-3-positive locations in Indonesia in 2005 (a) and 2019 (b) with areas marked in red indicating instances of CyHV-3 infection. Map illustration from Sunarto et al., (2005) (a) and WAHIS (2023).

² <https://wahis.woah.org/#/dashboards/country-or-disease-dashboard>

Table 3 : Koi herpesvirus disease worldwide.

Country	Disease Status	Reference
Europe		
Belgium	First outbreak 1999 - not confirmed. Outbreaks in 2002 & 2003 - confirmed by PCR. Reported known to be present	Jin et al 2010 www.cefas.defra.gov.uk/idaad . 2010
Czech Republic	First clinically apparent KHV infection	Novotny L. et al. 2010
Denmark	First outbreak in 2002 and two further in 2003 - confirmed by PCR. Reported known to be present	www.cefas.defra.gov.uk/idaad . 2010
Germany	Reported in country for the first time First outbreak in 1997 - over 80 cases in 2002 confirmed by PCR. Reported present or known to be present	Neukirch M, 2001 Jin et al 2020
Ireland	First detection of KHV in imported koi byPCR	www.cefas.defra.gov.uk/idaad . 2010
Luxembourg	Outbreak in 2003. Reported present or known to be present	Munich meeting, 2003. www.cefas.defra.gov.uk/idaad . 2010

Country	Disease Status	Reference
Poland	First detected in 2003 in carp Confirmed by PCR's, RT-PCR and <i>in situ</i> hybridization	Jin et al 2010
	Reported in country for the first time	Bergmann SM. Et al 2007
Romania	First reported occurrence of KHV	www.cefass.defra.gov.uk/idaad . 2010
Slovenia	First reported occurrence of KHV	www.cefass.defra.gov.uk/idaad . 2010
Spain	First reported occurrence of KHV	www.cefass.defra.gov.uk/idaad . 2010
Sweden	Reported present or known to be present	www.cefass.defra.gov.uk/idaad . 2010
United Kingdom	First reported occurrence of KHV First isolated in 2000 - suspicion in 1998 and 1999 - confirmed by PCR.	Walster C. 1999
Austria	First outbreak reported summer 2003 - confirmed by PCR.	.
France	Two suspicions in 2001; first confirmed outbreak in 2003	Munich meeting, 2003.
Italy	Strong suspicion by EM and Histology	Munich meeting, 2003.
Switzerland	First report in 2003 - suspicion before in 2001.	Centre for Fish and Wildlife Health, Munich meeting, 2003
America		
Canada	First detection of KHV by PCR	Garver KA. 2010
United States of America	First reported occurrence of KHV First outbreak 1998; virus isolated on KF cells. KHV diagnosed in ornamental carp. First outbreak in wild carp in Lake in May 2004.	Hedrick RP et al . 2000
Asia		
China	First detection of KHV by PCR KHV outbreak reported in 2001 in Hong Kong. handpicked koi positive in 2002 confirmed byPCR	Liu H, et al 2002 CEFAS Weymouth, UK.
China (Hong-kong)	Reported present or known to be present	
Taiwan	Reported in the country for the first time Reported present or known to be present	Tu C, et al . 2004 Sunarto A, et al 2005
Indonesia - Java- Sumatra & other islands	First outbreak reported in 2002; confirmed by PCR. Further outbreaks 2003 and spread to Bali, East Kalimantan and Sulawesi	Sunarto et al. 2005 Amin et al 2018
Israel	Reported in country for the first time First outbreaks in 1998 & outbreaks in all subsequent years	Hedrick RP et al 2000 Ariav et al. (1999), Perelberg et al. (2003)
Japan	Reported in the country for the first time First outbreak in May 2003; confirmed by PCR.	Sano M, et al . 2004
Korea	First report of KHV	Oh et al. 2001
South Korea	Outbreak in 1998,confirmed by <i>in situ</i> hybridization.	
Malaysia	Koi originating from Malaysia found positive by PCR in 2001 Reported present or known to be present	CEFAS, Weymouth, UK. CABI et al., 2022
Philippines	KHV associated mortalities in koi carp	Somga JR et al 2010
Singapore	Reported present or known to be present	

1.6.2 Molecular epidemiology

The first complete genome sequences of CyHV-3 were obtained for 3 strains isolated from outbreaks in the United States (KHV-U), Israel (KHV-I), and Japan (KHV-J) (Aoki et al. 2007). Sequence comparisons indicated a very high degree of identity (>99.9%) and showed that KHV-U was more closely related to KHV-I than to KHV-J. Probably because of the difficulty to obtain full genome sequences, genotype characterisation has long been done by identifying variations in the TK gene, which appeared as a suitable marker for samples from a similar geographic distribution (Han et al., 2013 ; Kurita et al., 2009; **Figure 14**). Epidemiological investigations were also made with distinct DNA markers (Bercovier et al., 2005 ; Cheng et al., 2011), among which ORF136 (Dong et al., 2011), or markers I (located between the ORF29 and ORF30 genes) and II (located close to the beginning of ORF133) (Bigarré et al., 2009). The marker I region displayed three alleles (I++, I+ and I-) whereas marker II region displayed two alleles: II+ in the Asian lineage and II in the European one (Bigarré et al., 2009 ; Sunarto et al., 2011). Eight markers of variable numbers of tandem repeats (VNTR) were also used to uncover genetic variations in CyHV-3 at a more intricate level (Avarre et al., 2011 ; Avarre et al., 2012). All these studies highlighted a notable genetic differentiation among the strains of CyHV-3, with a distinct separation between European and Asian variants, which were classified into two lineages (Kurita et al., 2009). These molecular markers are valuable for tracing strains, not just across continents, but also at a local level. However, such markers focusing on a few variable regions may not inform on the underlying whole genomic diversity. It was only in 2015 and 2016 that the next full genome sequences of CyHV-3 were published, either from isolated strains (Chen et al., 2015 ; Li et al., 2015) or directly from fish (Hammoumi et al., 2016). A comparison of these genomes with the reference ones published in 2007 confirmed the high level of identity and the existence of two lineages. Full genome sequencing was then applied to unveil other variations in CyHV-3 genomes and to compare the biological properties of the sequenced viruses (Gao et al., 2018 ; Klafack et al., 2019). These studies indicated that whole-genome sequencing is necessary to reveal the full diversity of the virus and better understand its origin and evolution.

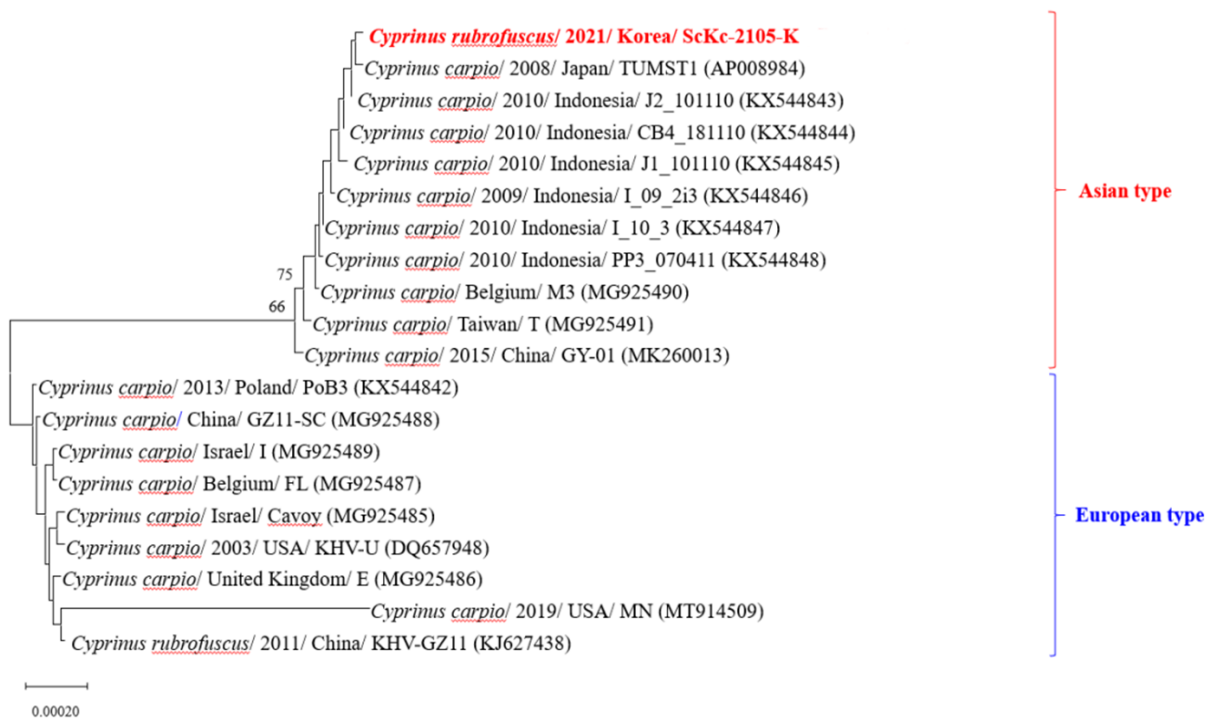


Figure 14 : A molecular phylogenetic analysis based on the partial nucleotide sequence of thymidine kinase gene obtained from the various strains of CyHV-3. Different colours denote specific lineages: Asian (red) and European (blue) (from Jeong et al., 2023).

1.7 Ecology of CyHV-3

1.7.1 Host range and potential reservoirs

The host range of CyHV-3 is currently limited to koi and common carp (Michel et al., 2010a), even though several studies have reported that CyHV-3 may cause disease to other related cyprinids, without clear evidence. It was shown, however, that different fish species can be carriers of CyHV-3 (El-Matbouli et al., 2007). Its genome has indeed been detected in many closely related species, such as goldfish, crucian carp, and grass carp (Bergmann et al. 2010). Initial laboratory trials suggest that CyHV-3 is safe for non-target species yet those fish may act as carriers or vectors (Sadler et al., 2008 ; Meyer et al., 2012 ; Kempter et al., 2009 ; McColl et al., 2017). CyHV-3 was also detected by PCR in Russian sturgeon (*Acipenser gueldenstaedtii*) and Atlantic sturgeon (*A. oxyrinchus*) from fish farms in Northern Poland (Kempter et al., 2009).

Vectors do not generally allow viruses to replicate, but they contribute to transmitting the pathogen (Jin et al., 2020). Surprisingly, zebrafish cells are inherently permissive to CyHV-3 replication (Streiff et al., 2023). Cross-species transmission was reported experimentally and in natural populations (El-Matbouli et al., 2007). The detection of CyHV-3 DNA and transmission to carp by cohabitation experiments has been reported in a wide variety of non-carp species from survey or disease trial studies, without the presence of clinical signs (Bergmann et al., 2020). Some carp species like fathead minnow (FHM) and goldfish are unlikely to be natural hosts for CyHV-3 and are not susceptible to KHVD (Tolo et al., 2021). CyHV-3 DNA was detected in at least one replicate qPCR test in brain and skin tissue of a single FHM, as well as in brain and gill of a single goldfish; but it did not indicate the presence of replicating CyHV-3, as evidenced by the lack of CyHV-3 mRNA detection by RT-PCR. The results suggested that the detection of CyHV-3 DNA in FHM and goldfish tissues originated from non-infectious CyHV-3 virions or naked DNA without evidence of replication or -disease. CyHV-3 was detected in swan mussels and scud using various PCR tests targeting viral genes, but there is no definitive demonstration of active multiplication of this virus within these animals (Kielpinski et al., 2010). Mussels might simply accumulate the virus passively. It appears these invertebrates can harbour the virus for extended periods. Another study examined wild duck intestines, and revealed that some samples showed positive for CyHV3 after amplifying a thymidine kinase gene fragment (Torres-Meza et al., 2020b). These findings provide further insights into the transmission of this virus to different aquatic animal species, where fish and ducks share common habitats. These results provide convincing evidence of the significant contagiousness of CyHV-3. CyHV-3 was detected in many fish as mentioned above. However, those are unlikely to be natural hosts for CyHV-3 and are not susceptible to KHVD. Actual susceptibility to CyHV-3 infection necessitates verification that indicates active viral replication, which can be observed through viral mRNA (Yuasa et al 2012 ; McColl et al., 2018 ; Tolo et al., 2023).

Cypriniviruses cause diseases only in their natural host species, which suggests the existence of restrictions related to host cell susceptibility (i.e., the ability to support virus entry) and host cell permissivity (i.e., the ability to support viral replication and the transmission of infectious viral progeny) (Rakus et al., 2019 ; Streiff et al., 2023).

1.7.2 Mode of transmission and persistence in natural environment

CyHV-3 is generally transmitted horizontally, though the possibility of a vertical transmission from koi to eggs has been suggested (Costes et al., 2009 ; Gomez et al., 2011). Horizontal transmission of CyHV-3 can be experienced through direct contact with skin lesions or the secretion of viral particles from mucus and urine in the same freshwater ponds (Costes et al., 2009 ; Rahmati-Holasoo et al., 2016 ; Negenborn et al., 2015). CyHV-3 can be introduced into a healthy fish population by adding infected fish to the same environment. However, direct contact between fish seems to be the primary mechanism of CyHV-3 transmission rather than through contaminated water (Tolo et al., 2022).

The virus remains infectious in water for at least 4 hours and even infectious for up to 3 days, explaining its highly contagious nature in ponds (Perelberg et al., 2003 ; Shimizu et al., 2006). Additionally, this virus remains infectious in the natural environment, such as plankton and suspended solids in the water (Minamoto et al., 2011). Surface water and sediment in a natural lake or pond serve as a potential reservoirs for CyHV-3 within natural freshwater environments (Minamoto et al., 2009b ; Honjo et al., 2012). CyHV-3 is distributed in freshwater, bottom sediments, mussels and freshwater shrimp, allowing it to infect healthy fish (El-Matbouli and Soliman, 2011 ; Kielpinski et al., 2010). Studies in Japan have also reported the detection of CyHV-3 DNA in plankton samples and, in particular, in rotifera species (Minamoto et al., 2011).

Several modes of experimental infection have been evaluated to observe CyHV-3 pathogenicity, like bathing the fish in virus-containing water (immersion) or cohabitation with sick fish. Bathing fish in virus-containing water caused 85 to 100% mortality within 7 to 21 days, and these mortalities were equivalent to those obtained after intraperitoneal injection. Infection of fish by bathing appeared to be more efficient than by cohabitation (Pikarsky et al., 2004). Carp from all ages are susceptible to CyHV-3 (Pikarsky et al., 2004 ; Cheng et al., 2011 ; Rakus et al., 2013 ; Cano et al., 2020). Fish handling, transportation stress and contaminated equipment (e.g., nets, tanks, and other tools used in fish handling) can also transmit the virus to healthy fish (Fabian et al., 2016 ; Minamoto et al., 2009b).

1.7.3 Temperature susceptibility

In line with other herpesviruses, CyHV-3 epidemiology is driven by seasonal changes in water temperature (Omori and Adams, 2011). In poikilothermic hosts, the severity and progression of all herpesvirus infections are affected by temperature, both *in vitro* and *in vivo*. It is generally acknowledged that CyHV-3 induces mortalities in farmed fish when water temperatures are comprised between 18 and 28°C (Pikarsky et al., 2004 ; Yuasa et al., 2008). Water temperature plays a role in the onset and severity of infection by altering virus replication and indirectly enhancing the efficacy of the host immune response. The optimal temperature range for replication of CyHV-3 is 16-23°C (Yuasa et al., 2008). Additionally, it significantly influences seasonal reproductive spawning aggregations, thereby affecting the recruitment of susceptible juveniles into the population (Joehnk et al., 2020). CyHV-3 induces mortalities in farmed fish when water temperatures are comprised between 18 and 28°C (Pikarsky et al., 2004 ; Yuasa et al., 2008). In temperate countries, CyHV-3 outbreaks occur mostly during spring and autumn, when the water temperatures rise above 20°C (Cheng et al., 2011). In contrast, outbreaks can occur all year round in tropical countries, such as Indonesia.

Shifting infected fish from a low to a permissive temperature (e.g. 22-23°C) rapidly induced mortality within 7–12 days (St Hilaire, et al. 2005 ; Pokorova et al., 2005 ; Cano et al., 2020). CyHV-3 becomes dormant, and clinical signs cease at temperatures above 30°C or below 13°C, (Gilad et al., 2003). Viral DNA synthesis is completely restrained at non-permissive temperatures, and CyHV-3 persists in fish maintained at low temperatures (12°C) for an extended period (St-Hilaire et al., 2005 ; Ilouze et al., 2012). CyHV-3 infection can be reactivated by temperature stress between 17°C and 23°C (Eide et al., 2011). Common carp exposed to CyHV-3 at 22°C showed a fast progression of the infection (Cano et al., 2020).

Temperature variations can cause changes in fish behaviour and physiological reactions, such as metabolism and stress response (Takahara et al., 2014 ; Abram et al., 2017). DNA of CyHV-3 and cortisol concentrations were higher under fluctuating temperature than under constant temperature (Takahara et al., 2014). Increasing water temperatures favour the pathogen or the host, depending on which is favoured (Marcos-López et al., 2010). The pathogen and the host each have an ideal temperature range for replication and immune response, respectively (Uchii et al., 2014 ; Abram et al., 2017). Delaying behavioural fever in fish could may boost viral replication and excretion by keeping infected fish at a suitable temperature (Ilouze et al.,

2012 ; Rakus et al., 2019). Additionally, it could promote viral transmission by encouraging physical contact by keeping infected fish at a temperature preferred by non-infected fish.

The α domain protein family of CyHV-3 (ORF112) exhibiting specific characteristics could restore viral replication, including the capacity to initiate liquid-liquid phase separation (LLPS) (Diallo et al., 2023). In the context of CyHV-3, this suggests that LLPS plays a crucial role in the life cycle of CyHV-3 to rescue viral replication in the absence of ORF112. Moreover, LLPS is highly sensitive to changes in temperature, and the relationship between temperature and the phase separation of biomolecules has been comprehensively investigated (Dignon et al., 2019). As the temperature increases, biomolecular interactions that drive LLPS become more dynamic, potentially modifying the composition and properties of the phase-separated compartments within the cell (Dignon et al., 2019). This temperature dependence of LLPS could have significant implications for the replication and spread of CyHV-3 within its host organism.

1.8 Pathogenesis of CyHV-3

1.8.1 Portals of entry in fish

The major mechanism of CyHV-3 transmission implies direct contact between fish (Tolo et al., 2022). The skin of carp has been proposed as the primary portal of entry of CyHV-3 (Costes et al., 2009 ; Adamek et al., 2013 ; Miwa et al., 2015), but CyHV-3 can also enter carp through infection of the pharyngeal periodontal mucosa after oral inoculation (Fournier et al., 2012). Therefore, direct transmission could result from skin to skin contact between infected fish and naïve ones, or from cannibalistic and necrophagous behaviours of carp (Fournier et al., 2012 ; Raj et al., 2011). Transmission of KHVD via skin-to-skin contact is more common during breeding in common carp (Uchii et al., 2014). Additionally, Tolo et al., (2021) found that direct transmission occurred in both disease phases (incubation and clinical), whereas indirect transmission was observed during the later clinical stage of the disease.

1.8.2 Cell entry and lytic replication of CyHV-3

CyHV-3 replication cycle starts when the envelope composed of glycoproteins is attached to the host cellular receptors. Viruses conduct penetration via fusion of envelope-membrane, then release the capsid and tegument content into the cytoplasm. The capsid is

transported to a nuclear pore via microtubules. Genomic viral DNA is then released and circularised into the nucleus of the host cell, where viral transcription and replication occur. Transcription of the viral genes is sequentially and coordinately regulated, resulting in immediate-early (IE), early (E), and late (L) viral mRNA synthesis (Ilouze et al., 2012b ; **Figure 15**). Cellular RNA polymerase II transcribes IE genes to generate regulatory proteins that are required for the expression of E genes. E genes encode the viral DNA replication machinery. *In vitro* studies showed that viral DNA synthesis initiates as early as 4 to 8 h post-infection (Ilouze et al., 2012a) and takes about 48 h (Davidovich et al., 2007). IE and E proteins, along with viral replication, are required for efficient late transcription, the protein products of which comprise the virion structure (capsid, tegument, envelope) or are involved in capsid assembly or virion budding processes. Structural components of the virion are translated in the cytoplasm and capsid proteins are then imported into the nucleus where they self-assemble and encapsidate the viral genome (Mettenleiter, 2004 ; Cheng et al., 2011). A primary envelopment occurs by budding of capsids through the nuclear envelope, out of which they are released into the cytoplasm by fusion of the primary envelope with the outer nuclear membrane (de-envelopment). In cytoplasm, naked capsids acquire the tegument and the glycoprotein studded envelope during a secondary envelopment into vesicles belonging to the trans-Golgi network, after which enveloped viruses are exocytosed as infectious virions (Miwa et al., 2007; **Figure 15**).

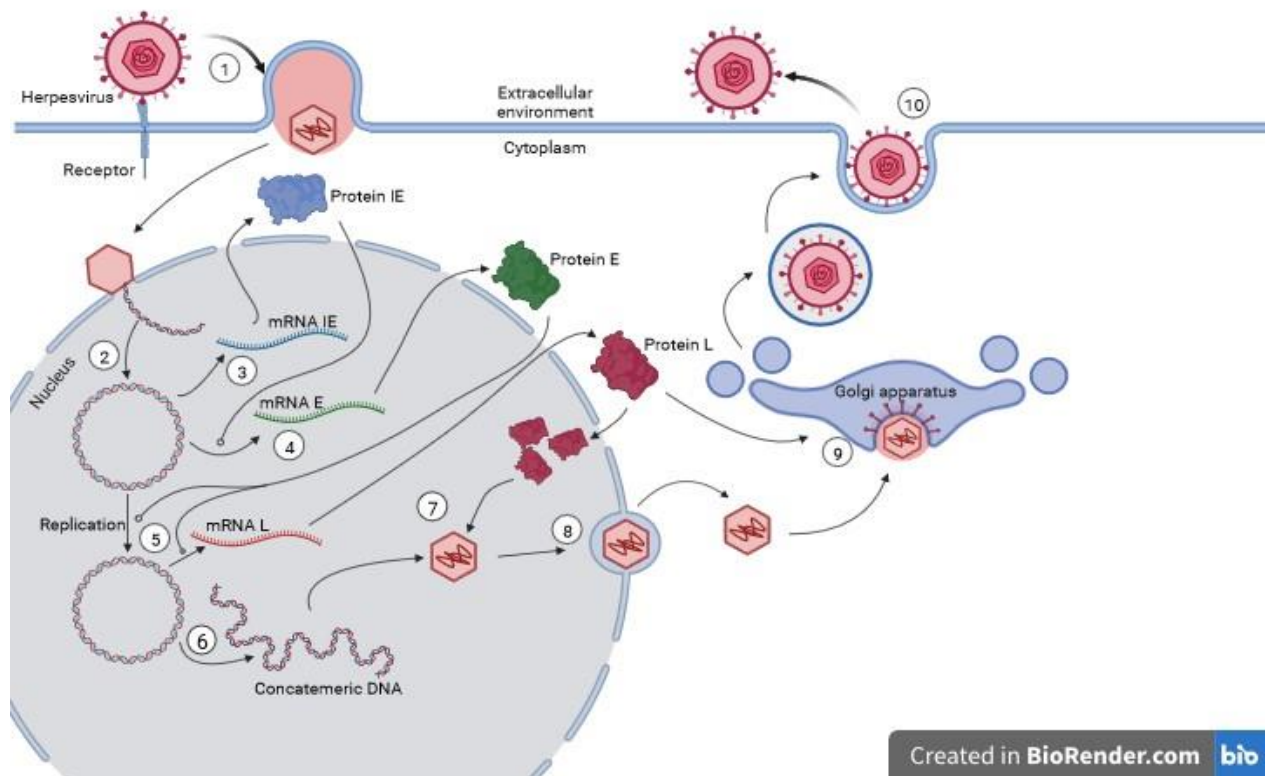


Figure 15 : Multiplication cycle of CyHV-3 (figure designed by F. Schatt with Biorender). 1: Adsorption and penetration, 2: Decapsulation, 3: The activation of IE genes, 4: The activation of E gene, 5: Transcription of late (L) viral gene expression, 6: Viral replication in a rolling circle and formation, 7: Assembly of capsid proteins and encapsidation 8: Budding into the nuclear envelope, 9: Acquisition of the tegument and budding into the Golgi apparatus, 10: Release of virions by exocytosis

1.8.3 Multiplication in target organs

High concentrations of CyHV-3 DNA and transcripts were detected in skin samples of carp at the early stages of infection (Adamek et al., 2012). For this reason, the skin has been proposed as a possible biopsy for non-lethal sampling to detect early infections, late infections and latent stages of CyHV-3 infections by real-time PCR (Adamek et al., 2013 ; Matras et al., 2012). The other target organs described for CyHV-3 are the blood, kidney, spleen, gut, and liver, as well as the fish intestine and intestinal tract (Dishon et al., 2007 ; Miyazaki et al., 2008 ; Eide et al., 2011 ; Fournier et al., 2012 ; Lee et al., 2016). CyHV-3 DNA was detected in the gonads and ovaries of koi broodstocks (Gomez et al., 2011 ; Rahmati-Holasoo et al., 2016). These findings indicated that the possibility of vertical transmission (from koi to eggs) of

CyHV-3 should not be dismissed. The most noticeable microscopic lesions are in the gills. The virus replicates in the diseased gills and is transferred through the white blood cells to the kidney (Pikarsky et al., 2004 ; Eide et al., 2013). These authors suggested that viral proteins are extensively expressed in kidneys of diseased fish and less in the brain and liver. These results agree with other experiments on diseased fish organs which showed that the kidney harbours large quantities of infectious viruses (Pikarsky et al., 2004). PCR of DNA from various tissues revealed that viral DNA is present in the blood and kidney of infected fish at very early times after infection, but appears in the brain, liver, and spleen only in the late stages of the disease (Pikarsky et al., 2004). The possibility that the virus replicates in the diseased gills and is then shed into the water is in agreement with the rapid and efficient spread of this contagious disease. From the gills, the virus is rapidly transferred to the kidney, where it resides in white blood cells and induces severe interstitial nephritis.

1.8.4 Clinical signs and histopathology

CyHV-3 infection shows similar clinical signs to systemic bacterial diseases. The most noticeable clinical signs of CyHV-3 infection are white patches, sunken eyes, spleen enlargement and mucus hypersecretion at the beginning of the infection (Bergmann et al., 2020; **Figure 16**). The first behavioural clinical signs are lethargy and reduced appetite. These initial signs of infection persist for 5-7 days, followed by a significant increase in mortality that occurs around the same time frame (Cheng et al., 2011). Infected fish also exhibit gill discoloration and necrosis (Ababneh et al., 2020). Signs of later stages of CyHV-3 infection are abnormal swimming behaviours, hyperemia on the fins and abdomen, pale patches on the skin, and peeling off in dead epithelium (Gomez et al., 2011 ; Rahmati-Holasoo et al., 2016 ; Ababneh et al., 2020). Morbidity can reach up to 100% within 2 or 3 weeks, appearing as clinical signs characteristic of an initial infection (Cheng et al., 2011 ; Hedrick et al., 2000).

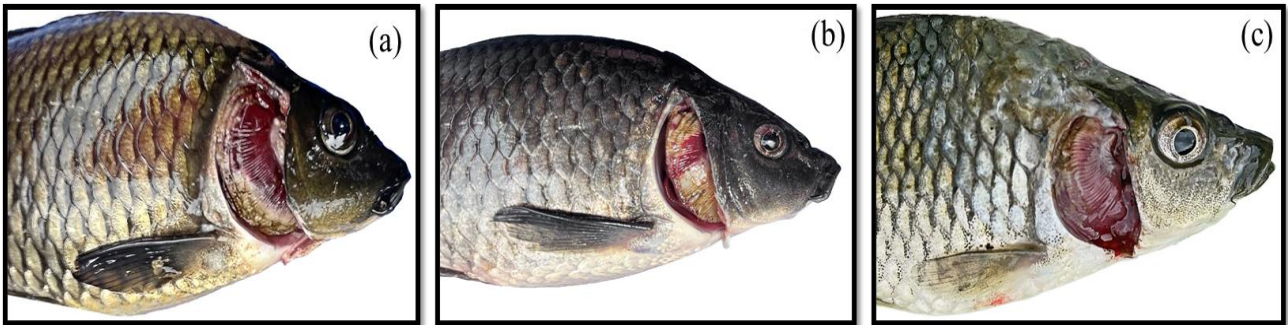


Figure 16 : CyHV-3-infected common carp. (a) Reddened skin, gill necrosis. (b) Discolouration of gills. (c) Hypersecretion of mucus in gills and body surfaces, gill swollen, gills and skin lesions (Personal own documentation).

Histological pathology includes acute hepatocellular necrosis, splenic necrosis, kidney necrosis, and hyperplasia of the secondary lamellae with focal necrosis (**Figure 17**). In many instances, the gills may be slightly swollen, with those lamellae close to the operculum being slightly pale in appearance. Mature virion was found in gills of infected fish, as depicted in (**Figures 18**).

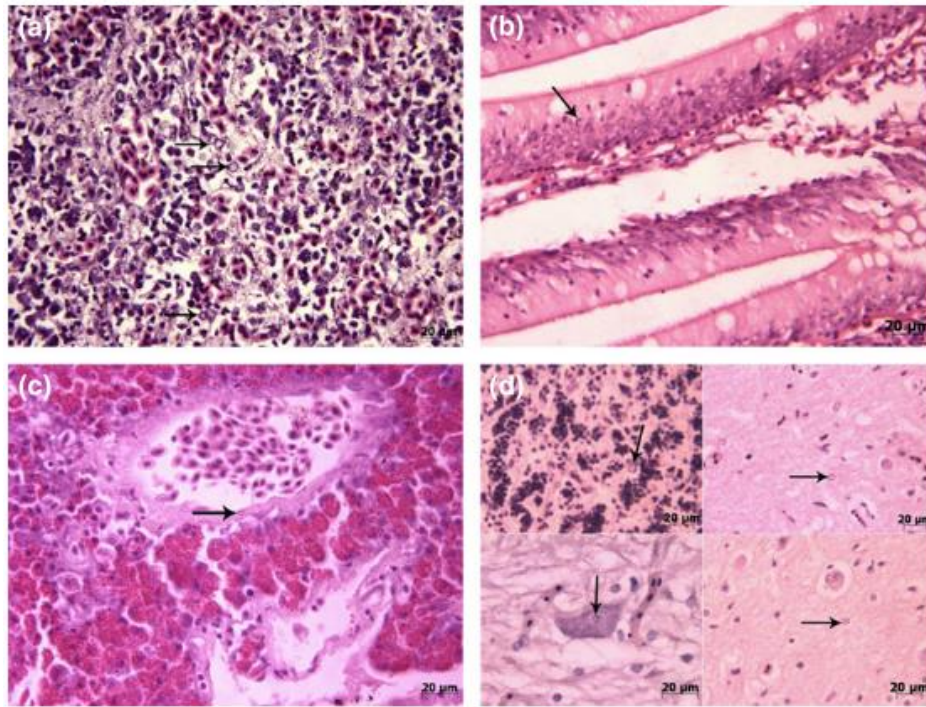


Figure 17 : CyHV-3-infected koi. (a) Respiratory epithelial cells of gill lamellae are fused with each other and many necrotic cells are seen at the fused lamellae. (b) In the gill filaments, respiratory epithelial cells exhibit necrosis (arrows) and intranuclear inclusion body (arrowhead). (c) In the kidney, the tubular epithelial cells show intranuclear inclusion bodies (arrowheads) and necrosis (bold arrow). Some of the renal tubular epithelial cells have a cloudy appearance (thin arrow). (d) In the ventricle of the heart, some myocardial cells display intranuclear inclusion bodies (arrows) (H&E) (from Rahmati-Holasoo et al., 2016).

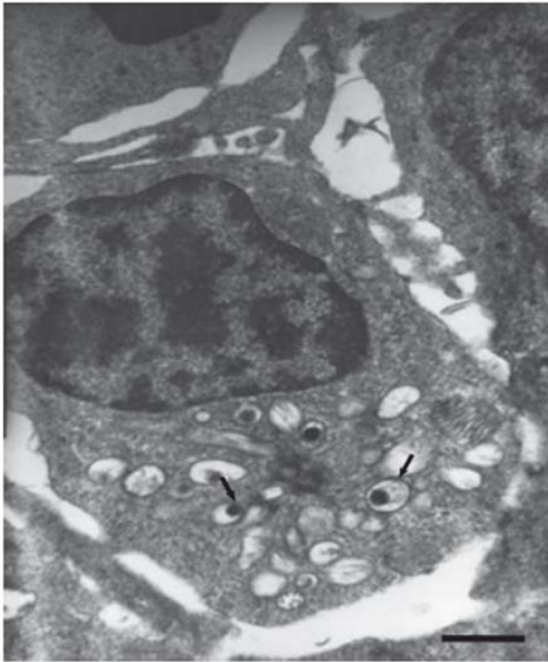


Figure 18 : Ultrastructural appearance of CyHV-3 particles in gills of CyHV-3-infected carp.

TEM indicates the presence of mature virions (arrows), each containing a loosely applied envelope and a round electron-dense core (bar = 640 nm) (from Cheng et al., 2011)).

1.9 Virus-Host interaction

Interaction between the host and virus is a dynamic interplay influenced by both host genetic factors and the genomic characteristics of the virus. Thus, variations in disease outcomes can be attributed to multiple factors, such as the developmental stages, availability of the host immune system and viral load (Monaghan et al., 2015). Carp of all ages can be affected by CyHV-3 infection, but carp sensitivity increases with the developmental stages (Ronsmans et al., 2014). Several carp strains with different genetic backgrounds have varying susceptibility to CyHV-3 infection (Rakus et al., 2009 ; Piačková et al., 2013). The virus may lack the capacity to replicate in resistant animals due to an incompatibility between the virus and the cellular receptor. Additionally, enhanced fish survivability can be associated with a more effective immune response that ceases infection progression (Wang et al., 2021) and lack of immunity to CyHV-3 can cause a 100% mortality rate in naive carp exposed to CyHV-3 (Ababneh et al., 2020).

1.9.1 Mucosal barrier disruption

CyHV-3 enters in fish through infection of the skin and/or the pharyngeal periodontal mucosa (Costes et al., 2009 ; Fournier et al., 2012). These mucosal epithelia play a key role in protecting against viruses (Gomez et al., 2013). They are covered by mucus that acts as a physical, chemical and immunological barrier against pathogens (van der Marel et al., 2012). It was showed that the skin mucus could inhibit CyHV-3 binding to epidermal cells (Raj et al., 2011) and that mucus removal treatment increases the sensitivity of carp larvae to CyHV-3 infection (Ronsmans et al., 2014), highlighting its critical role in protecting fishes against CyHV-3. Interestingly, Adamek et al., (2013) showed that CyHV-3 infection leads to the downregulation of mRNA expression of various genes encoding several important components of the skin barrier, in particular mucin 5B, antimicrobial peptides beta defensin 1 and 2, and the tight junction proteins claudin 23 and 30. This caused the disruption of the mucosal skin barrier resulting in changes in the bacterial flora and a higher susceptibility to secondary infections. The mechanism involving a probable virus-induced immunomodulation has not yet been elucidated.

1.9.2 Immune response to CyHV-3 infection

The importance of the innate and adaptive immune response in eradicating viral infections was highlighted by Hanson et al., (2011). Innate immunity is the first line of defence against invading pathogens. Innate immune responses are regarded as crucial in the immune system of fish (Baloch et al., 2023). They rely on the recognition of conserved features of pathogens, called Pathogen-associated Molecular Patterns (PAMPs), that stimulate inflammatory responses and phagocytosis by cells such as neutrophils and macrophages. Cells infected with viruses produce interferons (IFNs), which induce a series of cell responses to inhibit viral replication, activate the natural killer cells and initiate specific adaptive immune responses. Type I IFNs are thus recognized as the initial line of defence against viral infections.

Several studies have reported various aspects of innate immune responses during a CyHV-3 infection in common carp (Adamek et al., 2013 ; Pionnier et al., 2014 ; Rakus et al., 2009 ; Syakuri et al., 2013). *In vivo*, CyHV-3 infection induces a systemic IFN type I response in carp skin, intestine and head kidney, that is correlated with the virus load (Adamek et al., 2013 ; Syakuri et al., 2013). Furthermore, innate immune response constrains the spread of

CyHV-3 by limiting viral replication. Infection is then quickly controlled, with rapid viral clearance associated with apoptosis-like death of infected cells (Streiff et al., 2023). Transcriptomic analyses of CyHV-3-infected larvae revealed upregulation of interferon-stimulated genes, in particular those encoding nucleic acid sensors, mediators of programmed cell death and related genes (Streiff et al., 2023). They also highlighted a correlation between differences in resistance to CyHV-3 and differential expression of immune-related genes (Rakus et al., 2012 ; Hwang et al., 2017 ; Machat et al., 2022). Significantly higher expression of several immune-related genes including those that are involved in pathogen recognition, complement activation, MHC-I restricted antigen presentation and development of adaptive mucosal immunity was detected in the more resistant carp lines (Rakus et al., 2012).

Adaptive immune responses are carried out by lymphocytes and are composed of two broad classes: the antibody response and cell-mediated response. The antibody immune response to CyHV-3 infection has been assessed by measuring anti-CyHV-3 antibodies in the serum of infected carp (Adkison et al., 2005 ; Perelberg et al., 2008 ; Ronen et al., 2003 ; St-Hilaire et al., 2009). Detection of anti-CyHV-3 antibodies starts at 7-14 dpi, increases to 20-40 dpi, then gradually decreases to 150 dpi (Perelberg et al., 2008 ; Ronen et al., 2003), and correlates with protection against CyHV-3. Interestingly, temperature strongly affects the adaptive immune response of fish with a reduced immunocompetence in low temperature conditions (<14°C) (Abram et al., 2017 ; St-Hilaire et al., 2009). CyHV-3 can influence hosts' behaviour by expressing a single gene (ORF12) which may act as a TNF- α , a mediator of behavioural fever in ectotherms (Rakus et al., 2019). The study underscored that the expression of behavioural fever occurred at 32°C, manifesting in zero mortality. Conversely, when this behaviour was absent, mortality reached 100%

1.9.3 Latency of CyHV-3

Latency is a common feature among all herpesviruses. It corresponds to the persistence of the viral genome in host cells for the life of the host, in the absence of productive infection and viral replication, ensuring the maintenance of its genetic information (Roizman and Pellet, 2001; Minarovits et al., 2007). During latency, the virus genome circularises and persists as an episome (Boss et al., 2009). CyHV-3 latency sites have been investigated in several tissues of fish that had recovered from an infection (Eide et al., 2011 ; Gilad et al., 2003). CyHV-3 DNA

was detected in the brains of fish that survived to primary infection and showed no clinical signs, as late as 1 year post-infection (St-Hilaire et al., 2006 ; Miwa et al., 2015 ; Yuasa & Sano, 2008). Viral DNA was consistently found in white blood cells of koi surviving to CyHV-3, which suggests that white blood cells are the preferred latency site for CyHV-3, specifically the B lymphocytes (Eide et al., 2011 ; Reed et al., 2014). Limited genes are actively transcribed during latent infection; detection of pORF6 in B cells from latently-infected koi suggests the putative role of this viral protein in latency (Reed et al., 2015 ; Reed et al., 2017)).

CyHV-3 can persistently infect farmed (Baumer et al., 2013) or wild carp populations (Uchii et al., 2014) was shown to persist in wild populations of common carp for at least two years after an initial outbreak, with the ability to be transmitted to naïve fish (Shimizu et al., 2006 ; Minamoto et al., 2012 ; Xu et al., 2013 ; Panicz et al. 2022). Furthermore, the success of CyHV-3 in infecting the host is inherently related to its apparent ability to cause persistent infections (Uchii et al., 2014 ; Zheng et al., 2017 ; Amin et al., 2018). Persistence, or latency, has implications concerning the control and eradication of the disease because hosts appear healthy but may transmit the virus to naïve carp.

Various stressors, including temperature changes, inadequate water quality, food scarcity, and overcrowding, can compromise the physiology and the immune system of *C. carpio*, potentially leading to the reactivation of viral processes (Shimizu et al., 2006 ; Leung and Bates, 2013 ; Omori and Adams, 2011 ; Tolo et al., 2021)). As mentioned earlier, temperature strongly affects the adaptive immune response of fish (St-Hilaire et al., 2009 ; Abram et al., 2017 ; Scharsack and Franke, 2022), and consequently plays an essential role in inducing latency and reactivation of CyHV-3.

CyHV-3 DNA, but not virions, was detected in infected fish maintained for 64 days post-infection at non-permissive temperatures (Haenen et al., 2004; Gilad et al., 2004). It was shown that viral DNA and infectious viruses persist for approximately 200 days in infected fish maintained at non-permissive low temperatures (12°C) (St-Hilaire, et al., 2005). Fish with latent CyHV-3 infection can live normally; however, they can excrete infectious virus and succumb to viral disease when they undergo latent reactivation under stressful conditions, such as increased temperature (Gilad et al., 2004 ; Miyazaki et al., 2008 ; Lin et al., 2017). The dynamic interaction between virus-host depicted in **Figure 19**. Temperature of the water can thus regulate the switch between latency and lytic replication, allowing the virus to persist in the

host population throughout the seasons even when the temperature is non-permissive for the outbreak.

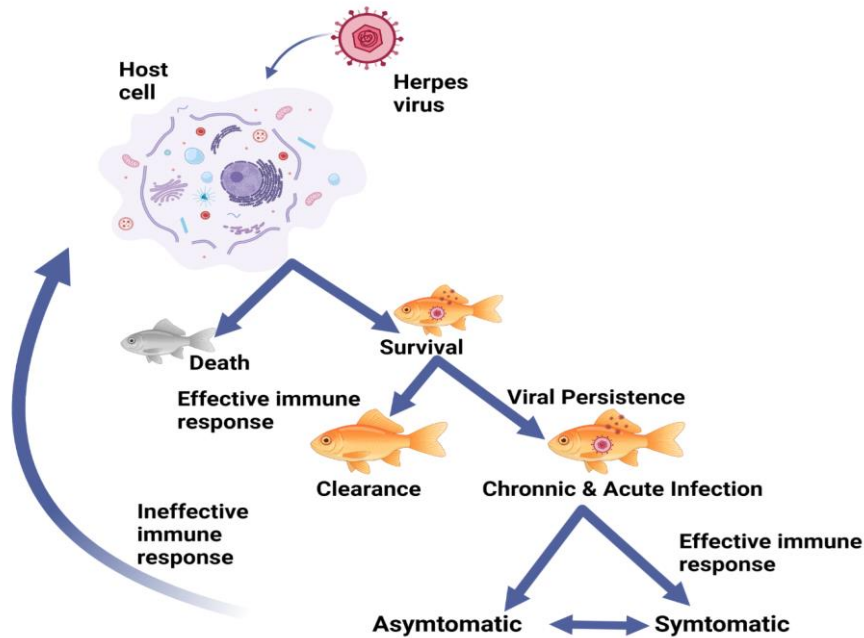


Figure 19 : The dynamic interactions between a virus-host for the establishment and maintenance of persistent infections.

1.10 Virus multiplication in cell culture

1.10.1 Permissive cell lines

Virus inoculation to cell cultures permits valuable studies of infectious aquatic viruses, as it allows to amplify infective virus particles (Monaghan et al., 2016 ; Li et al., 2021). CyHV-3 has been the object of many studies through cell culture propagation for characterising its biological characteristics (Adamek et al., 2012 ; Tu et al., 2014). Various cell types are used to propagate this virus, such as common carp brain (CCB) cells, koi fin (KF) cells, koi caudal fin (KCF-1) cells or cells derived from gills like KoG (Dong et al., 2011 ; Lin et al., 2013). An exhaustive list of the cell lines permissive or not to CyHV-3 replication is available in (Boutier et al., 2015b). CyHV-3 has also the ability to infect cell lines derived from species within the

family *Cyprinidae* that are not its natural hosts, even if inefficiently infecting (Rakus et al., 2019).

Primary cultures of KF and KCF-1 cells were primarily used to isolate the etiologic agent of KHVD in Israel (Perelberg et al., 2003 ; Ronen et al., 2003). However, among all cell types available in culture for carp, CCB cells are the most permissive cells for CyHV 3. The CCB cell line was reported to be more stable and able to produce higher viral titers than other cells (Davidovich et al., 2007; Mletzko et al., 2017). Several factors could link to the failure of cell culture propagation, such as the absence of optimal cell surface receptors, leading to inefficient viral entry. Viral entry might occur, but the absence of essential cellular components could hinder the initiation of viral replication (Streiff et al., 2023). The difficulty of culturing CyHV-3 is most likely due to the relative fragility of its virions (Davidovich et al., 2007). Indeed, many factors influence a viral infection, particularly the abiotic conditions such as pH, temperature or buffer capacity. These factors have to be considered in the cell culture system to maintain favourable conditions and obtain the optimum yields. Varying serum content in cell culture medium inoculated with CyHV-3 proved the stabilising effect of proteins in preserving its infectivity (Tolo et al., 2021).

Virus growth in the CCB cell line was optimal at temperatures between 18 and 25°C and undetectable at 30°C (Rakus et al., 2017). The highest virus concentrations at 20°C were observed after 7 days of culture (Gilad et al., 2003). The virus was detected at 4°C and 10°C at the very beginning of infection, but after two days, no CyHV-3 DNA was detected, and there was also no evidence of virus growth at 30°C or 37°C during *in vitro* propagation (Gilad et al., 2003).

1.10.2 Cytopathic effects

CyHV3 rapidly produces cytopathic effects (CPE) on cultures of CCB cells, generally between 2-4 days at 20-25°C (Tu et al., 2014). Fuchs et al., (2011) reported that CPE could be seen after four days of infection in CCB cells with a multiplicity of infection (MOI) of 0.01. For comparison, using KF-1 cell cultures, CPE appeared within 7-10 days post-inoculation (Hedrick et al., 2000), suggesting that CCB are better suited for *in vitro* studies. The appearance of CPE consists in the formation of syncytia with increasing cell volume and development of abundant endoplasmic vacuoles (Pikarsky et al., 2004; **Figure 20**). Finally, infected cells become rounded before detaching from the substrate. In this scenario, infected cells continue

to exhibit metabolic activity for viral replication. CPE-related changes may also involve modifications in growth rate, kinetics, and specific metabolic functions.

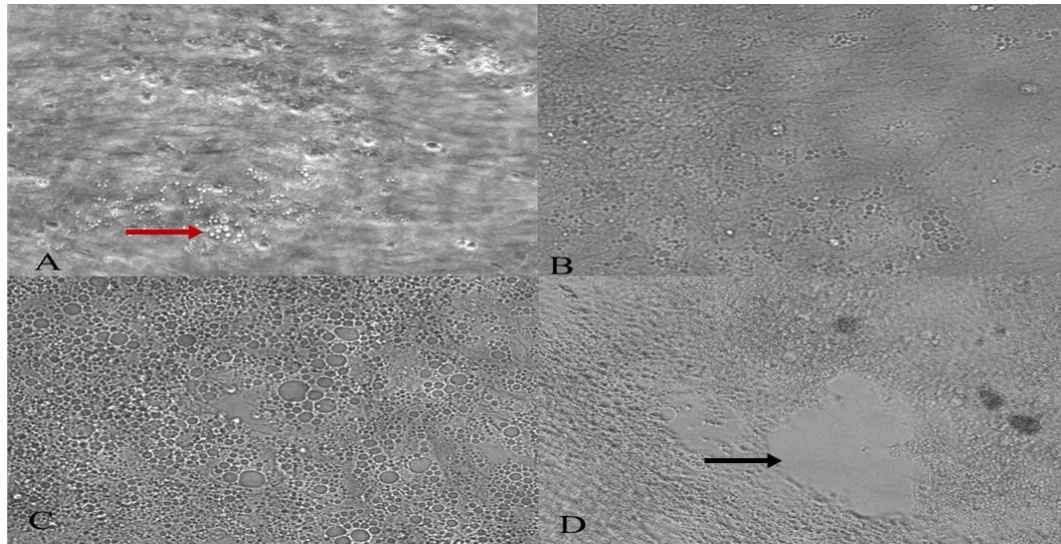


Figure 20 : CCB cells in MEM at 24°C infected with CyHV-3. CCB cells in MEM at 24°C infected with CyHV-3. Images were taken (A) 2 days, (B) 3 days, (C) 4 days and (D) 6 days after virus inoculation. The first CPE signs of small plaque formation appear 2 days post inoculation (red arrow). Cytoplasmic vacuolation is visible for infected cells in C. Black arrow showed that CyHV-3 developed syncytium (D). Personal light microscopy images (10X magnification).

1.11 Diagnostic tools

As for other aquatic viruses, the rapid global spread of CyHV-3 urged the design of practical surveillance tools. Several molecular diagnostic tools were quickly made available for the detection and characterisation of CyHV-3, e.g., fluorescence *in situ* hybridisation (FISH), enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (Kurita 2009 ; Garver et al., 2010 ; OIE Aquatic Manual, 2022; **Figure 21**). The virus can be detected directly after amplification in cell cultures, but virus isolation is rarely successful, particularly when examining samples from fish with subclinical infections. Samples must be collected promptly after the fish's death. Additionally, the sensitivity and accuracy of the PCR assay rely on the selection of the DNA extraction techniques, primer set, sample preservation, and DNA polymerase used (Engelsma et al., 2013). Not all infected fish could be consistently detected

with all methods, and significant variations in sensitivity were observed among these techniques. Reliable diagnostics for early detection of the virus is vital to prevent false-negative results, which can be detrimental when screening fish prior to transportation or during surveillance programmes (Cho et al., 2014 ; Colorio et al., 2020). The PCR tests could detect CyHV-3 in apparently healthy or carrier fish (Cho et al., 2014). Acutely infected fish undergoing clinical disease could be successfully diagnosed using the majority of the developed molecular tests (Bercovier et al., 2005).

Detection of thymidine kinase (TK) gene by PCR was listed by WHOAH as a reliable diagnostic method (WOAH, 2022). TK gene is well-conserved and has been successfully used for CyHV-3 diagnostics (Bercovier et al., 2005 ; Bergmann et al., 2010). The sphI-5 primer set developed by Yuasa et al. (2005) was slightly more sensitive than the TK primer set developed by Bercovier et al. (2005) and was less susceptible to genomic variations (Hedrick et al., 2005 ; OIE 2012 ; Monaghan et al., 2014). However, several reports have indicated that single-step PCRs have some limitations in detecting fish with low viral copy numbers (Bergmann et al., 2009 ; Monaghan et al., 2015) or during latent, persistent infections (Grimmett et al., 2006 ; Bergmann et al., 2010 ; Vrancken et al., 2013). Even though high throughput sequencing (HTS) is becoming a gold standard in molecular diagnostics of viral infections, it is still facing the same limits as PCR regarding sensitivity (Nkili-Meyong et al, 2017 ; Avarre, 2020).

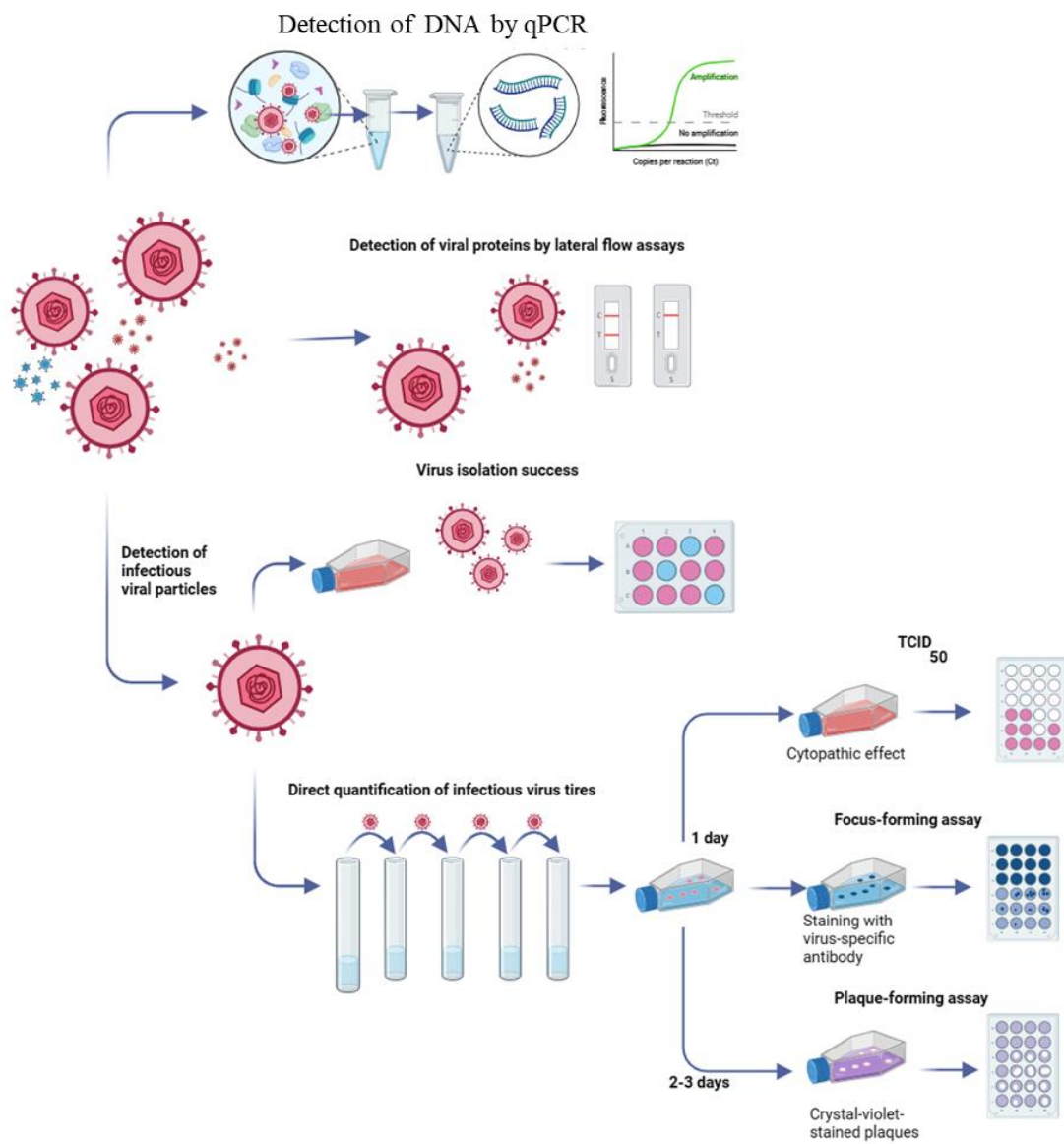


Figure 21 : A review of recent progress in the detection methods and identification of CyHV-3 (figure designed with Biorender).

1.12 Controlling Koi herpesvirus disease

A comprehensive risk assessment is warranted to limit global dissemination across continents. Prevention through proper biosecurity measures includes quarantine procedures (a minimum of 4 weeks to 2 months before mixing with naïve fish) and health monitoring (Taylor et al., 2011). Most efforts to control CyHV-3 outbreaks in cultured ponds are still relying on the use of chemicals for the disinfection of ponds, farm equipment and eggs (Kasai et al., 2005 ; Amtmann et al., 2020) or antiviral medications (Troszok et al., 2021). However, chemical drugs only help overcome secondary infections by bacteria, fungi or parasites (Watts et al., 2022 ; Nik Mohamad Nek Rahimi et al., 2022).

1.12.1 Virus inactivation

CyHV-3 can be inactivated by UV radiation at a dose of $4.0 \times 10^3 \mu\text{Ws}/\text{cm}^2$, temperatures above 50°C for 1 minute and by iodophor (200 mg/L), benzalkonium chloride (60 mg/L), ethyl alcohol (30%) and sodium hypochlorite (200 mg/L) treatments (Kasai et al., 2005). Temperature manipulation has also been applied to eradicate the CyHV-3 from its host. They exposed carp to CyHV-3 at 30°C, where the virus became inactive. This process allows carp carrying the inactive virus to develop adaptive immunity against it. Nonetheless, this intervention is expensive and typically results in losing 30–40% of the fish stock during operation. Additionally, this practice can sometimes trigger outbreaks due to the release of the virus into the environment (Ronen et al., 2003 ; Lin et al., 2017). In addition, carp treated under this strategy become asymptomatic or symptomatic again after stopping the treatment (Tolo et al., 2021). It has been suggested that this control strategy may suppress the epidemic but not stop it completely.

1.12.2 Selection of resistant carp strains

Other attempts have been made to prevent the risk of CyHV-3 infection, including selection of resistant carp strains (Tadmor-Levi et al., 2017). These approaches encompass techniques like hybridising various carp or selection of resistant carp through genetic selection using potential genetic markers for subsequent breeding programs of common carp (Rakus et al., 2009 ; Adamek et al., 2019).

1.12.3 Vaccination

Apart from the necessary biocontrol measures, vaccination seems to be the most favoured way to control KHVD, even though, to date, there is no safe and licenced commercial vaccine available in major parts of the world. The principle of the vaccination is to stimulate a protective immune response to control or suppress infections. Vaccination strategy requires attentive consideration of factors such as the type of vaccines, fish size targeted, farming system type and the administration or delivery protocol. A number of vaccine approaches have been tested against KHVD, including inactivated vaccines (Schmid et al., 2016), modified live attenuated vaccine (Weber et al., 2014), subunit vaccine (Zhou et al., 2017 ; Ma et al., 2020), or DNA vaccine (Liu et al., 2018), attenuated recombinant vaccine (Ilouze et al., 2010 ; Boutier et al., 2017 ; Klafack et al., 2022 ; Schröder et al., 2019),

Live-attenuated vaccines have been the earliest ones to control KHVD developed by serial passages in cell culture (Ronen et al., 2003 ; Perelberg et al., 2005). It shows high efficacy and cost-effectiveness. Live attenuated vaccines closely mimic a natural viral infection within the host (Bøggwald and Dalmo, 2019). These vaccines typically maintain their innate ability to enter the host without requiring specialised delivery methods, and stimulate the immune system by their replication in the host. Thus, vaccine strains of this nature are well-suited for mass vaccination in aquaculture, such as large-scale inoculation of fish through bathing. Due to their remarkable effectiveness, attenuated viral vaccines have arguably stood out as one of the most significant and efficient methods to combat the spread of viral diseases (Boutier et al., 2019). Conventional anti-CyHV-3 attenuated vaccine was developed by serial passages in cell culture and UV irradiation and commercialised in Israel and Indonesia by KoVax Ltd. for the vaccination of koi and common carp (Perelberg et al., 2008 ; Ronen et al., 2003). This vaccine, launched on the US market under the name Cavoy, was withdrawn from sale after just a year, partly due to residual virulence for fish weighing less than 50 g (Perelberg et al., 2008 ; Weber et al., 2014), and uncertainties about its preparation and unforeseen risks. Indeed, the origin of the attenuation is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded (Meeusen et al., 2007 ; Gao et al., 2018). It has been proposed that while this control strategy may suppress the outbreaks, it might not completely stop it.

Carp subjected to this approach may become asymptomatic during treatment and raise safety concerns. Indeed, the residual virulence and the possible reversion or recombination to a

more virulent phenotype of attenuated vaccine may lead to virus spreading to naïve individuals (Schmid et al., 2016 ; Ma et al., 2020). Syncytial plaque formation in cell culture is a common characteristic among CyHV-3 strains, and this genetic trait is associated with the SNP, either an alanine (183A) or a threonine (183T) residue in ORF131. Experiments involving the recombinant viruses observed that SNP has exclusively differed, and 183A genovariant displayed more excellent fitness in vitro but reduced fitness in vivo (Gao et al., 2023). Interestingly, during simultaneous infections with both variants, the less fit variant was quickly removed in both lab and living conditions, indicating a natural selection process favouring the more fit variant. This process was accelerated by viral interference at the cellular level and host resistance to superinfection.

Schröder et al. (2018) demonstrated efforts to develop safe and effective attenuated recombinant vaccines by focusing on modifying non-essential genes of CyHV-3. Genetic modification of the virulence-related genes of CyHV-3 led to the development of recombinant attenuated virus strains that enhance host immunity against KHVD. Vaccine constructions targeting various ORFs have been tested, including ORF16, ORF55, ORF123 and ORF134, which encode a G protein-coupled receptor (GPCR), thymidine kinase (TK), deoxyuridine triphosphatase (dUTPase) and interleukin-10, respectively (Costes et al., 2008 ; Fuchs et al., 2011 ; Ouyang et al., 2013). However, disruption of the thymidine kinase locus induces partial attenuation in koi (Costes et al., 2008). A vaccine candidate based on the double deletion of ORF56 and ORF57 was produced using BAC cloning technology, and the effectiveness of this latter has been demonstrated in experimental challenge experiments (Boutier et al., 2015b ; Boutier et al., 2015a). In the study of Klafack et al. (2022), an attenuated recombinant virus deleted in ORF150, that might code for an ubiquitin E3 ligase, was shown to protect carp or koi after immersion or oral application compared to wild type challenge. Another study used chitosan-alginate capsules as an oral delivery method for a live recombinant probiotic vaccine expressing the KHV ORF81 protein via *Lactobacillus rhamnosus* (Huang et al., 2021). This encapsulated probiotic vaccine remains highly viable and tolerant in diverse digestive environments. It depicted satisfactory immunogenicity, inducing a significant level of antigen-specific IgM and effective CyHV3-neutralizing activity.

DNA and subunit vaccines are reported to have the potential to effectively treat CyHV-3 infection. The selection of target genes (proteins) is essential in the formulation of DNA and

subunit vaccines. The envelope protein is essential because it facilitates viral entry, morphogenesis, and release. Additionally, its ability to stimulate neutralizing antibodies makes it ideal as a vaccine candidate. The DNA vaccines consisting of plasmids encoding viral antigens, such as the type-I membrane proteins from the ORF25 family (ORF25, ORF148, ORF149), the glycoprotein ORF81 as well as the capsid proteins ORF72 and ORF92 are strong vaccine candidates that showed efficient results under lab conditions (Hu et al., 2020 ; Zhou et al., 2014 ; Schröder et al., 2019b). One promising subunit vaccine was developed in Japan, by the use of a liposome-based vaccine containing CyHV-3 particles inactivated by formalin. Oral administration was effective in protecting carp against clinical disease, achieving 70% surviving carp after challenge (Yasumoto et al., 2006). More recently, Liu et al., (2020) published their results on a combined DNA and protein subunit vaccine, based on ORF131 of CyHV-3, achieving less than 80% protection. Using a single antigenic protein gene as a DNA vaccine may not provide sufficient protection across various challenge models. Embregts et al. (2019) demonstrated robust protection in fish that received three injections of either of the two DNA vaccines. DNA vaccines have been tested by directly applying intraperitoneal injection (Zhou et al., 2014). Administering the vaccine via the intramuscular route is unsuitable for mass vaccination on a farm because it is labour-intensive and expensive. Additionally, the process can potentially handle the stress that causes death in vaccinated fish. Injectable DNA vaccines have demonstrated effectiveness in controlled laboratory settings, but they have practical limitations, such as the low value of individual common carp and the need for vaccination at a very young age.

Vaccine efficacy can be influenced by the administration mode, and closely mimicking the natural infection route may offer higher reliability. The pharyngeal periodontal mucosa was recognized as the primary entry site following oral exposure, while the skin functioned as the primary entry point following immersion exposure (Fournier et al., 2012). Oral administration is a more promising immunisation route for fish at all life stages, including the larval one, and is easy-to-use. Different oral immunisation approaches have been formulated to protect the antigen from gastrointestinal digestion. Some pleasing alternative strategies that could lead to enhanced vaccine effectiveness are bio-encapsulation in a natural starter feed (artemia), using the *Saccharomyces cerevisiae* cell surface display of CyHV-3 envelope protein pORF65, or chitosan-alginate-based encapsulated vaccines (Su and Su, 2018 ; Embregts et al., 2019). Oral

vaccines of recombinant lactic acid bacteria encoding the CyHV-3 ORF81 have been tested in experimental stages against CyHV-3 infections (Cui et al., 2015 ; Huang et al., 2021). Detailed information about the vaccine's components, including genes or proteins, allows for better vaccine design and optimization. Eventually, vaccine efficacy can vary significantly depending on type of vaccines, the preparation method and the route of administration.

1.12.4 Antiviral agents and immunostimulation

Antiviral agents are used to inhibit virus replication. Acyclovir (ACV), a deoxyguanosine analog, which is capable to halt viral replication via selective inhibition of the viral DNA polymerase, and Cidofovir, an acyclic phosphonate nucleotide analog, are approved antivirals for the treatment of Herpes Simplex Virus 1 and 2 in humans and hence good candidates for use in pet fish and fish farming. These drugs firmly bind to the viral thymidine kinase, inhibiting viral DNA synthesis and terminating herpesvirus infections. Acyclovir as well as its tricyclic derivative, have previously been studied for their effectiveness against CyHV-3 infection, both in laboratory settings and in live animal experiments (Troszok et al., 2019 ; Sosa-Higareda et al., 2022 ; Matsuoka et al., 2023). Quijano Cardé et al., (2020) has evaluated the efficacy of these two antivirals in decreasing cumulative mortality during a CyHV-3 challenge in koi fingerlings. The results indicated that only acyclovir (for a 10 mg/kg intracoelomic dose) was capable of reducing cumulative mortality, by a mean of 15%, and suggested that multiple doses are probably required for effective treatment of fish. Treatments with antiviral drugs, however, are largely ineffective because they reduce the replication of the virus but not its transmission (Kausar et al., 2021 ; Sosa-Higareda et al., 2022).

The carp aquaculture sector urgently requires safe, cost-effective and sustainable methods for the prevention and control of KHVD, essential for the sustainable development of fish breeding. A convenient solution would be a cheap medicinal molecule that could be orally administered in feed during virus reactivation seasons. Naturally occurring antiviral substances are derived from plants, mushrooms, or their components. Several medicinal plants have been studied extensively to evaluate their biological activities, including anticarcinogenic, anti-inflammatory and antimicrobial activities (Liao et al., 2022a). Plants have many active ingredients in flavonoids, phenols, terpenoids, steroids, and essential oils (Citarasu, 2010 ; Thitinarongwate et al., 2021). Hence, they function as antimicrobials, antioxidants, anti

stressants, appetite enhancers, and immunostimulants (Chang et al., 2012 ; Reverter et al., 2014). To date, few plant extracts were designed against viruses; for instance, administration of *Gracilaria tenuistipitata* extract by immersion or orally could enhance the immune response and resistance of shrimp against the white spot syndrome virus (Sirirustananun *et al.*, 2011 ; Lin et al., 2011). Some active compounds from medicinal plants contained in N-hexane, dichloromethane, or methanol extracts, inhibited the attachment and penetration of herpesvirus *in vitro* (Cheng et al., 2004 ; Vachirayonstien et al., 2010 ; Kunsorn et al., 2013). In addition, phenolic, polyphenol, terpene, flavonoid and sugar-containing compounds from other medicinal herbs were also reported as exhibiting anti-HSV activities (Astani et al., 2011 ; Chen et al., 2011). Their effects on CyHV-3 have been studied *in vitro* by evaluating antiviral activity of Lindau extracts (Haetrakul et al., 2018).

Fritzsche et al., (2021) reported that microalgae and cyanobacteria may be promising sources of natural bioactive substances against CyHV-3. *In vitro* and experimental infection show that exopolysaccharides (EPS) from marine algae have antiviral activity against CyHV-3 (Reichert et al., 2017 ; Bergmann et al., 2022). EPS not only inhibit CyHV-3 replication, but also enhance immune response, probably as an immunostimulant. Polysaccharides have an enormous potential for future as additives for aquaculture. Due to their antimicrobial activity, lactic acid bacteria and their post-metabolites have been tested *in vitro* as candidates in prophylaxis and therapy for CyHV-3 infection (Vilhelmova-Ilieva et al., 2023). Yoshida et al., (2013) used anti-CyHV-3 bacteria isolated from the intestinal contents of carp and from carp habitat water to control CyHV-3 outbreaks and assumed an antiviral effect of these bacteria

1.13 Genetic diversity and evolution of CyHV-3

Genetic diversity of viruses plays a major role in viral evolution, pathogenesis, immune escape, and drug resistance. Understanding the mechanisms that shape the genetic variability and evolution of viral populations is crucial for devising effective strategies of disease control, antiviral drug development, and vaccine design. It could also be useful in limiting or even predicting viral outbreaks. Different mechanisms are responsible for genetic variability, including replication errors, genome editing, recombination and segment reassortment in the case of segmented viral genomes. This diversity is subsequently modulated

at the viral population level by natural selection and random genetic drift, depending on multiple demographic and ecological factors (Sanjuán and Domingo-Calap, 2021).

1.13.1 Sources of genetic diversity

The mutation rates of viruses vary according to genome composition (RNA or DNA), size, and structure (single- or double-stranded). The mutation rates for RNA viruses are in the range of 10^{-6} to 10^{-4} base substitutions per nucleotide and per cell infection whereas these rates range from 10^{-8} to 10^{-6} in DNA viruses. Single-stranded viruses appear to mutate faster than double-stranded viruses, and similarly to what has been shown previously for DNA viruses, there appears to be a negative correlation between mutation rate and genome size among RNA viruses (Sanjuán and Domingo-Calap, 2021).

Production of error copies underlies quasispecies dynamics which is crucial for virus adaptation to changing environments (Domingo et al., 2019). Viral quasispecies are the mutant distributions (also termed mutant clouds) that are generated upon replication of RNA viruses, and some DNA viruses in infected cells and organisms (Lauring and Andino, 2010 ; Domingo et al., 2012). Viral quasispecies are currently defined as collections of closely related viral genomes subjected to a continuous process of genetic variation, competition among the variants generated, and selection of the most fit distributions in a given environment (Domingo et al., 2012).

The main mechanisms responsible for introducing variations in viral genomes include replication errors, lack of proofreading, repair avoidance, diversity-generating elements, host-encoded editing of viral genomes, recombination and segment reassortment (**Table 4**).

Table 4 : Sources of genetic diversity in seven major virus classes. Source of diversity generally present (+), present in some cases (+/-), or not shown or infrequent (-) (from Sanjuán and Domingo-Calap, 2021).

	dsDNA	ssDNA	Para retro	Retro	dsRNA	(+)RNA	(-)RNA
Lack of 3' exonuclease proofreading	-	+/-	+	+	+	+	+
Avoidance of post-replicative repair	-	+/-	+	+	+	+	+
Use of error-prone repair polymerases	+/-	+/-	-	-	-	-	-
Diversity-generating retro-elements	+/-	-	-	-	-	-	-
APOBEC hyper-mutation	+/-	+/-	+	+	-	-	-
ADAR hyper-mutation	-	-	-	-	+/-	+/-	+/-
Reassortment	+/-	+/-	-	-	+/-	+/-	+/-
Template switching	-	-	+	+	+	+	-
Recombinases	+/-	-	-	-	-	-	-

Apolipoprotein B mRNA editing catalytic polypeptide-like enzymes (APOBECs) function as an innate immune response against reverse-transcribing viruses. Double-stranded RNA-dependent adenosine deaminases (ADARs) constitute another type of host-encoded enzymes capable of editing and hypermutating RNA virus genomes.

In herpesviruses, sources of genetic diversity include mainly mutations, intra-genomic rearrangements and inter-genomic recombinations (Pérez-Losada et al., 2015 ; Thiel et al., 2015 ; Karamitros et al., 2018). Single nucleotide mutations (SNPs) generally arise from replication errors, even in the presence of proofreading and DNA repair mechanisms, characteristics of large DNA viruses (Renner and Szpara, 2018 ; Pandey and Szpara, 2019 ; Wilkinson and Weller, 2003 ; Poss, 2011 ; Duffy, 2018). For illustration, the mutation rate in HSV-1 has been estimated to be around 6×10^{-8} substitutions per nucleotide and per cell infection (Drake and Hwang, 2005 ; Sanjuán and Domingo-Calap, 2016).

Genomic rearrangements, or structural variations (SVs), correspond to deletions, insertions, duplications and inversions, with a minimal length of 30 to 50 nucleotides (Emerson and Cook, 2020). SVs appear to be a widespread evolutionary mechanism in DNA viruses, enabling them to adapt to changing environments (Poirier and Vignuzzi, 2017 ; Vignuzzi and

López, 2019 ; Loiseau et al., 2020). Those located in tandem repeats can be produced as a result of "polymerase stuttering", which leads to the same repetition being replicated several times. Other structural variations may be due to recombination mechanisms. Genetic recombination could be defined as the exchange of genetic material between two DNA or RNA segments. Recombination is a widespread phenomenon in viruses and a more complex process in DNA viruses than in RNA viruses. Recombination involves specific host or viral recombinases and seems highly frequent in some dsDNA viruses, such as α -herpesviruses (Robinson et al., 2011 ; Thiry et al., 2005) which encode their own recombination systems. In herpesviruses, recombination is intimately linked to DNA repair and replication (Weitzman et al 2004), and particularly favoured by the rolling circle replication mode of herpesviruses (Pérez-Losada et al., 2014). The α -herpesviruses example is probably the best characterised one, as these viruses represent an important model for the study of eukaryotic DNA replication (Weller and Coen, 2012 ; Pérez-Losada et al., 2015). Recombination is sequence-dependent, and the regions containing multiple repeats, like the terminal repeats, are highly prone to unequal crossovers. Recombination can be homologous, when it occurs in the same site in both parental strands, or non-homologous or illegitimate, when it occurs at different sites of the viral genomes (Pérez-Losada et al., 2015). Homologous recombination is the most frequent mechanism in herpesviruses although illegitimate recombination is also observed (Umene, 1999). Inter-genomic recombination occurs when at least two viral genomes co-infect the same host cell and exchange genetic segments of their genomes (Spinard et al., 2023 ; Weller and Sawitzke, 2014). In this case, recombination can prevent the progressive accumulation of deleterious mutations in viral genomes to maintain fitness; it can also facilitate access to evolutionary innovations, provided that the genomic structures of the recombinants are not aberrant.

CyHV-3 harbours the largest genome of all known herpesviruses, and with no exception, it undergoes the same mechanisms of genome evolution including mutation, intra-genomic rearrangement and inter-genetic recombination (Lee et al., 2015 ; Gao et al., 2018). The interplay of these molecular alterations, along with natural selection, genetic drift and co-evolution with hosts, is essential in the diversity and adaptability of herpesviruses, including CyHV-3, over time.

1.13.2 Evolution of viral populations

Evolution of viral population is defined as a change in the frequency of alleles (or variants) in a viral population over time. The allele frequency is the rate at which a specific allele appears within a population. Four main forces can drive change in allele frequencies: mutation, natural selection, genetic drift (the random change in allele frequencies), and gene flow (the transfer of alleles between populations).

The fate of new genetic variants is largely determined by selection. The frequency of a new variant in the viral population will tend to increase, fluctuate randomly, or decrease depending on whether it has a beneficial, neutral, or deleterious effect on viral fitness, respectively. The frequency can also change as a result of random genetic drift. Drift is determined by the effective size of the viral population, which in turn depends on spatial structure, population size bottlenecks during host-to-host transmission, viral growth dynamics, and so on. Both selection and genetic drift are conditioned by multiple demographic and ecological factors, which results in a variety of evolutionary processes at the population level (**Figure 22**) (Sanjuán and Domingo-Calap, 2021).

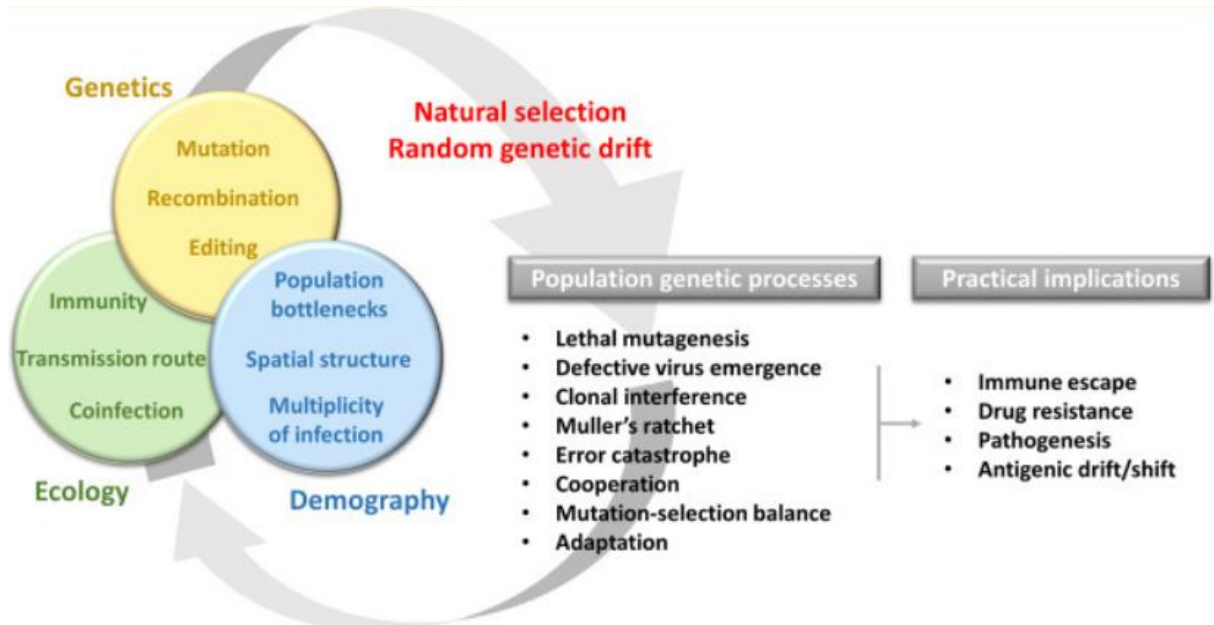


Figure 22 : Genetic, demographic and ecological processes determining viral population diversity. Mutation is the ultimate source of genetic variation, but recombination also produces new genotypes. Ecological and demographic processes determine how natural selection and random genetic drift acts on these new genetic variants. This leads to a variety of possible population genetic processes, which in turn feedback on viral demography, ecology and genetics. Immune escape, drug resistance, and pathogenesis are a visible consequence of such processes. The list of factors shown is not exhaustive (Sanjuán and Domingo-Calap, 2021).

The diversity of herpesviruses can be maintained through transmission or reduced by genetic bottleneck, geographic spread, natural selection and antiviral treatment (Boyer et al., 2011 ; Koelle et al., 2017). Genetic bottlenecks imply a severe reduction in diversity between hosts that could turn to the loss or gain of some genetic variants (Akhtar et al., 2019). These bottlenecks depict points of genetic shrinkage, where the diversity of viral populations can be significantly reduced. Furthermore, **Figure 23** (modified from an original article by (Renner and Szpara, 2018) illustrates the evolutionary course of viral populations as they undergo and pass through these bottlenecks. This visualisation allows us to comprehend how genetic diversity evolves under different bottleneck conditions, whether in a controlled laboratory environment or a living organism. Certain modifications in the genome can lead variants to thrive and dominate within the viral population (Leung et al., 2017). Selection is imposed by the host's immune system, environmental factors and antiviral treatments (Hwang et al., 2004).

The extent of selective pressure exerted on the virus in its host environment can significantly shape the accrual of mutations (Szpara et al., 2014).

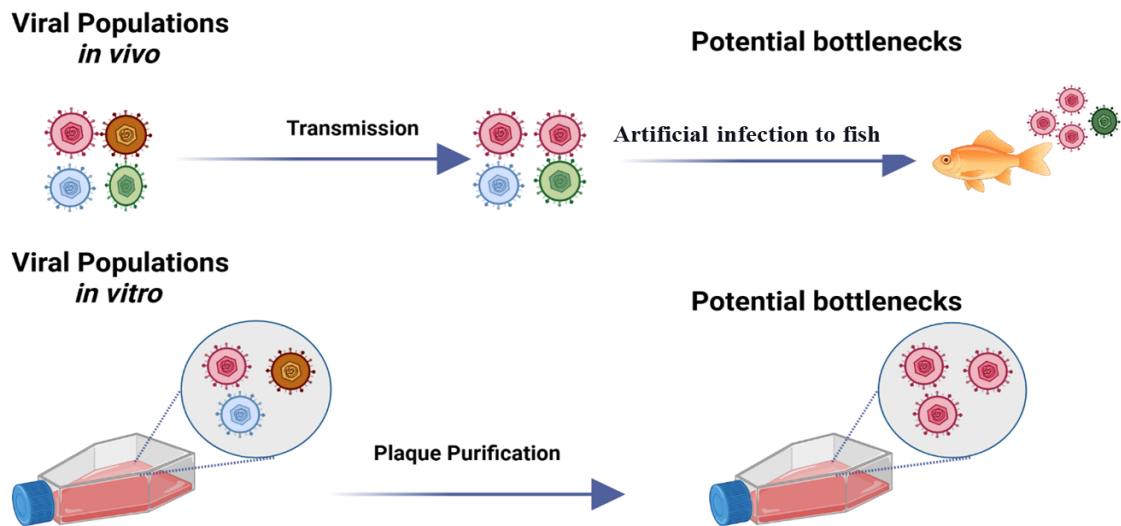


Figure 23 : Bottleneck during the transfer to a different host or propagation to cell culture can create opportunities for novel variants to gain dominance (Illustration is adapted from (Renner and Szpara, 2018), with some modifications)

Diversity can lead to negative (competitive) or positive (cooperative) interactions between variants in the case of coinfection in the same cell. In turn, these interactions feedback on diversity by modifying the population frequency of certain variants.

Defective viral genomes (DVG) are produced by illegitimate recombination during high viral multiplication. These defective viruses lack large portions of the genome and can only replicate thanks to the help of the complete viral genomes present in the same cell. This process becomes prevalent when cells are infected by multiple virus particles for several infection cycles, leading to frequency-dependent selection that affects the fitness of DVGs. In addition to replicating faster than the wild type because their genomes are shorter, defective viruses often interfere with some steps of the infectious cycle. Defective viral genomes have been mostly reported in RNA viruses (Manzoni and López, 2018), yet they may also accumulate during herpesvirus infections due to a combination of random errors during viral replication at high viral titers (O'Hara et al., 1984 ; Molenkamp et al., 2000 ; Vignuzzi and López 2019). CyHV-

3 infections have been shown to be the result of genotype mixtures, both *in vivo* and *in vitro* (Hammoumi et al., 2016 ; Klafack et al., 2019). Such mixed-genotype infections are likely to represent an important source of diversification for CyHV-3 on the one hand and to confer resilience to DVGs and genetic drift, on the other hand (Renner and Szpara, 2018 ; Delmotte et al., 2020).

Coinfection of cells with different virus variants may also allow cooperative interactions. Two viruses each carrying a different genetic defect can mutually increase their fitness values if they share gene products (genetic complementation). It is also possible that each variant has optimised a different aspect of the infection cycle, and that such variants achieve higher fitness in coinfection than alone (Sanjuán and Domingo-Calap, 2021).

Thus, the diversity generated by genetic drift is subject to constant fitness-based selection. This selection is dictated by environmental pressures, which may differ markedly *in vitro* (in cell culture) and *in vivo* (in the natural host) (Gao et al., 2023).

1.13.3 Slow evolution of CyHV-3

CyHV-3 isolates may carry mutations in ORFs that are likely to alter gene functions, and these mutations vary from virus to virus (Gao et al., 2018). ORFs containing one or more frameshifts at the beginning or within the central region are prone to disrupting protein function. In contrast, ORFs with a frameshift closer to the end might not necessarily impact protein function (Gao et al., 2018). Aoki et al (2007) hypothesised that virulent CyHV-3 would have arisen from a wild-type ancestor by loss of gene function. However, nearly 15 years later, this hypothesis has still not been tested, probably because of the lack of extensive genome-phenotype investigations between isolates. As mentioned previously, the few comparisons of CyHV-3 full genome sequences highlighted two main lineages that share a very high degree of identity (>99.9%). Therefore, more than twenty-five years after its emergence, and in spite of all its available “tools” for diversification, this virus seems to have evolved at a slow pace. Considering its high geographical distribution, the picture is probably not so simple. There are two possible explanations for this : 1) genomic comparisons mostly focused on single nucleotide polymorphisms (SNPs), which are not representative of the whole genomic diversity, as outlined above; 2) such comparisons were mainly carried out on consensus

sequences. If it artificially reduces the actual diversity of a viral population, it also only takes into account the major variations.

Major and minor variations in a viral population are present at a frequency above and below 50%, respectively (Allison et al., 2014 ; Delmotte et al., 2022). The accurate description of viral genetic diversity (including major and minor variations) depends on several parameters, such as the quantity and quality of viral genetic material, sequencing quality and depth, as well as the bioinformatics tools used (Sanjuán and Domingo-Calap, 2019). For instance, detecting structural variations using short-read sequencing has limited the ability to reconstruct repetitive sequences and detect complex structural variations. Long-read sequencing has overcome several limitations of short-read sequencing regarding the detection of structural variations (Suvage et al., 2018).

1.13.4 Next-generation sequencing (NGS) and virus evolution

Next-generation sequencing, including second and third generations, are now frequently applied for monitoring outbreaks and for genome surveillance purposes in aquatic environments. Genome surveillance plays a crucial role in aquaculture, such as confirming suspected disease cases and controlling the spread of disease (Stärk et al., 2019). Another advantage is to provide insight into developing disease control, such as an eradication program or demonstrating the absence of disease or infection within a specific population (Peeler and Otte, 2016).

Sequencing of a specific viral marker, like a gene, has proven very useful to understand the ways a virus propagates between aquaculture settings, as exemplified with the infectious pancreatic necrosis virus (Mutoloki et al., 2016). It is also an excellent means to uncover the evolution of a virus over time, especially when relevant epidemiological data is available (Abbadi et al., 2016). In comparison with specific amplicon-based sequencing, whole genome sequencing (WGS) enables to resolve micro-evolutionary distances, but also to discover new and rare variations, including those that may arise during an outbreak or that evolve *in vivo* during an infection (Bayliss et al., 2017). However, WGS is still facing many challenges when applied on environmental samples, such as fish or water. For instance, recovering full viral genomes, especially large double-stranded ones, require a sufficient read depth that even the

third generations of sequencers cannot provide (Nkili-Meyong et al., 2016 ; Avarre, 2020). For recovering the full genome sequences of salmonid alphavirus (SAV) from several salmon tissue samples, (Gallagher et al., 2020b) amplified its genome with a set of 6 primer pairs prior to sequencing. If this is possible for small viruses, it is yet not suitable for large ones, such as CyHV-3. Moreover, this strategy is not compatible with studying genetic diversity and evolution at a fine scale. For this reason, the same authors have developed a specific target-enrichment method, which they applied to study the intra-host diversity of SAV (Gallagher et al., 2020a). Their results revealed an unexpectedly high diversity of this virus in farmed and wild fish populations.

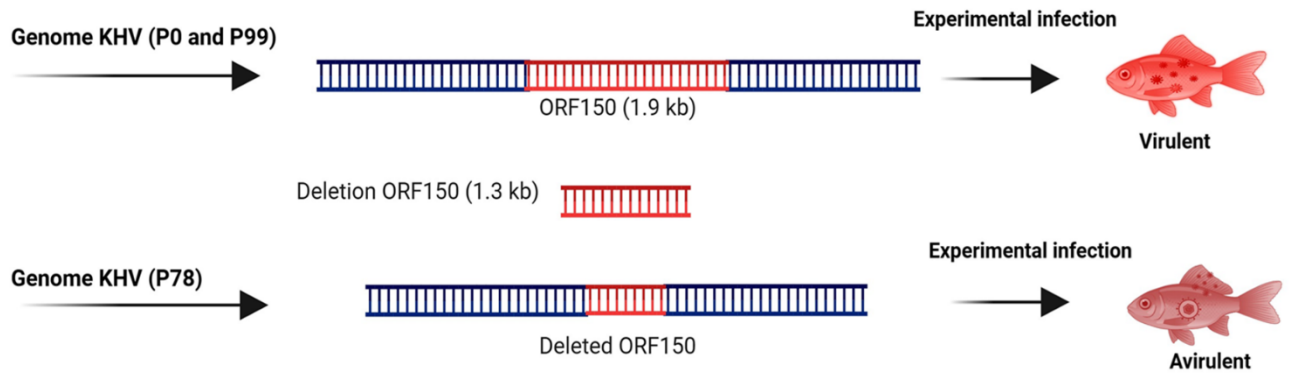
In the context of CyHV-3, genomic surveillance using NGS is still challenging. Using a specific target enrichment strategy, Hammoumi et al., (2016) could obtain for the first time sequence datasets with 100% genome coverage directly from fish. If this strategy is very useful to estimate the extent of genetic diversity within and between samples, it remains costly to study CyHV-3 spread and evolution over time. The use of cell cultures may overcome this limitation for obtaining full-length genomes. Discovering full-length sequence viral genomes *in vitro* may be useful to understand virus evolution. The utilisation of advanced sequencing technologies enabled to detect and describe diversity occurring during serial transmission, encompassing various types of mutations, including intricate structural variants (Bai et al., 2019). Sequencing technologies have revealed that most clinical and laboratory-grown isolates of herpesvirus undergo extensive genome alterations. Comparisons of full-length CyHV-3 sequences with biological properties *in vitro* and *in vivo* have enabled to draw relationships between the evolution of this virus and its consequences, *e.g.* in terms of adaptation (Gao et al., 2018) or of virulence (Klafack et al., 2019). In this latter study, a deletion of 1,363 bp occurring in the ORF150 could be associated with a decrease in virulence. Surprisingly, this deletion was detected after 78 consecutive passages, but not after 99 passages (**Figure 24**). This analysis was performed with second-generation sequencing.

Combining *in vitro* studies with long read sequencing technology has enormous potential to elucidate the mechanisms underlying viral evolution. Third generation sequencing technologies, such as Oxford Nanopore, enable direct, real-time analysis of short to very-long DNA or RNA fragments, in a fully scalable format. They provide several advantages over second generation, including minimal sample preparation, no need for clonal amplification and

long read lengths (Feng et al., 2015 ; Avarre, 2020). The Oxford Nanopore MinION technology measures changes in the electronic current as single molecules of DNA pass through biological pores (Jain et al., 2016). This current disruption is subsequently interpreted using a base-calling algorithm to decipher the DNA sequence. This approach offers a powerful tool to study and understand the intricate processes that drive genetic variation and adaptation in controlled laboratory environments, thereby shedding light on the broader mechanisms in natural environments.

A

Short-read sequencing



B

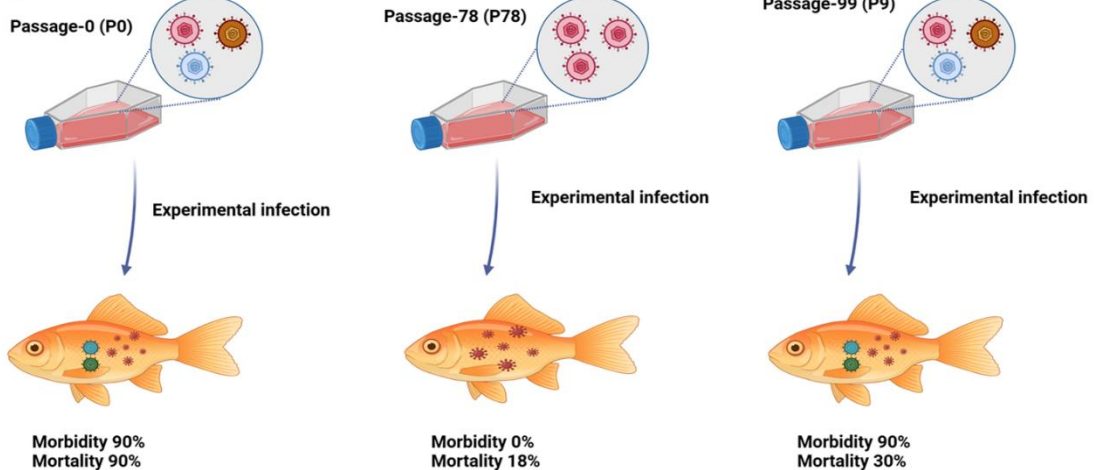


Figure 24 : Genotype-phenotype association using in vitro cell culture and next-generation sequencing. (A) The comparative analysis of different passages revealed a deletion of 1,363 bp in the ORF150 after 78 passages, but not after 99 passages. (B) Experimental infections with P0, P78 and P99 had significantly different outcomes: P78 elicited a significantly reduced mortality and morbidity in comparison with the original isolate (P0), whereas P99 exhibited an intermediate phenotype (Klafack et al., 2019).

2. Research questions and objectives

As presented in the previous section, CyHV 3 infections are commonly caused by a mixture of haplotypes, both *in vivo* and *in vitro*. *In vitro*, the composition of such haplotype assemblies may quickly vary along successive infectious cycles, resulting in quick phenotypic modifications (Klafack et al., 2019). This context was the basis of my PhD work, which was organised around three main objectives:

The first objective of this work was to get more insights into the evolutionary mechanisms underlying such haplotype assemblies in cell culture (CCB cells).

The second objective aimed at investigating the prevalence of ORF150-del and other structural variations in this genomic region *in vivo*, through the sampling and the study of carp populations in Indonesia.

The third objective consisted in exploring the accumulation pattern of structural variations and single nucleotide polymorphisms in the entire CyHV-3 genome during serial passages in cell culture and under thermal stress conditions.

Each of these questions is addressed in the following chapters, in the form of an article. Chapters 1 and 2 correspond to published articles, whereas chapter 3 displays a manuscript that is still in construction. These three chapters are followed by a general discussion that seeks to explore the evolutionary mechanisms of CyHV-3 and their consequences, and to propose future trends of research, in particular in view of combating this virus more effectively.

3. Chapter 1 - Structural variation turnovers and defective genomes: key drivers for the in vitro evolution of the large double-stranded DNA koi herpesvirus (KHV)

Context

This first chapter is a continuation of an earlier study carried out on a CyHV-3 isolate from Taiwan (Klafack et al, 2019). After 100 consecutive passages on CCB cell culture, experimental infections with viral populations of passage 78 (P78) and passage 99 (P99) revealed different levels of virulence as compared to the original isolate: P78 was significantly less virulent, whereas P99 had an intermediate virulence profile. Short-read sequencing of these different passages revealed a deletion of 1,363 bp in the ORF150 after 78 passages, but not after 99. If these findings showed a potential connection between the phenotype and genotype, with ORF150 likely significantly influencing CyHV-3's virulence, they primarily highlighted that the proportions of particular haplotypes can quickly vary from one infectious cycle to the other.

These findings laid the basis of the present study, to better comprehend the mechanisms that led to the presence and absence of this 1363-nt deletion during cell passages. Using the same isolate, we expanded the analysis using ultra-deep whole-genome sequencing to investigate structural variations (SVs) within the CyHV-3 genomic mixture at P78 and P99. In order to trace the unexpected dynamics of gain and loss of the full ORF150 along passages, we also searched for the SV turnovers during ten intermediate passages (P10, P20, P30, P40, P50, P70, P80 and P90) by employing amplicon-based sequencing.




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Structural variation turnovers and defective genomes: key drivers for the *in vitro* evolution of the large double-stranded DNA koi herpesvirus (KHV)

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ABSTRACT

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Structural variations (SVs) constitute a significant source of genetic variability in virus genomes. Yet knowledge about SV variability and contribution to the evolutionary process in large double-stranded (ds)DNA viruses is limited. Cyprinid herpesvirus 3 (CyHV-3), also commonly known as koi herpesvirus (KHV), has the largest dsDNA genome within herpesviruses. This virus has become one of the biggest threats to common carp and koi farming, resulting in high morbidity and mortalities of fishes, serious environmental damage, and severe economic losses. A previous study analyzing CyHV-3 virulence evolution during serial passages onto carp cell cultures suggested that CyHV-3 evolves, at least *in vitro*, through an assembly of haplotypes that alternatively become dominant or under-represented. The present study investigates the SV diversity and dynamics in CyHV-3 genome during 99 serial passages in cell culture using, for the first time, ultra-deep whole-genome and amplicon-based sequencing. The results indicate that KHV polymorphism mostly involves SVs. These SVs display a wide distribution along the genome and exhibit high turnover dynamics with a clear bias towards inversion and deletion events. Analysis of the pathogenesis-associated ORF150 region in ten intermediate cell passages highlighted mainly deletion, inversion and insertion variations that deeply altered the structure of ORF150. Our findings indicate that SV turnovers and defective genomes represent key drivers in the viral population dynamics and *in vitro* evolution of KHV. Thus, the present study can contribute to the basic research needed to design safe live-attenuated vaccines, classically obtained by viral attenuation after serial passages in cell culture.

Keywords: KHV, virus evolution, virulence, virus attenuation, structural variations, defective genome, carp

Introduction

Viruses have a remarkable ability to adapt to the complex and hostile host immune and physiological constraints. Such capability is directly associated with the viral population's genetic diversity, and deep characterization of this diversity is the cornerstone of our understanding of virus evolutive response to a new cellular environment. The starkly evident examples are RNA viruses. These viruses, such as HIV, hepatitis C, and influenza, display high mutation rates which generate significant polymorphism levels, allowing the viral population to quickly adapt to newly infected cellular environments and evolve strategies against vaccines and antiviral drugs (Lauring and Andino 2010; Loiseau et al., 2020). However, the genetic diversity has been thoroughly characterized in only a handful of viruses, mainly targeting SNPs (Single Nucleotide Polymorphisms) in RNA viruses (Sanjuán and Domingo-Calap 2016). Although the mutation rate of large double-stranded (ds) DNA viruses is up to four folds lower than that of RNA viruses, due to the use of high-fidelity proofreading polymerases, and most SNPs in dsDNA viruses are neutral and at low frequency, SNP-based approaches were chosen to analyze the genetic diversity in Human cytomegalovirus (HCMV), *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and herpes simplex virus 2 (HSV-2), for example (Renzette et al., 2015; Chateigner et al., 2015; Akhtar et al., 2019).

Structural variations (SVs) play a key role in viral evolutionary processes. Genome rearrangements such as deletions, insertions, duplications and inversions can lead to defective viral genomes (DVGs) (O'Hara et al., 1984; Molenkamp et al., 2000; Vignuzzi and López 2019). Preben von Magnus first identified DVGs in the late 40's as incomplete influenza viruses that can interfere with the wild-type virus replication (Vignuzzi and López 2019). Since then, the role of DVGs in antiviral immunity, viral persistence and their negative impact on virus replication and production has been established (Bull et al., 2003; Li et al., 2011; Vignuzzi and López 2019; Loiseau et al., 2020). Nowadays, DVGs have been described in most RNA viruses and to a lesser extent in dsDNA viruses (Vignuzzi and López 2019; Loiseau et al., 2020). Despite the critical role of SVs in virus infection dynamics, the knowledge about structural variation diversity, and their evolutionary impact in viral populations, especially those with large dsDNA, is limited.

The large dsDNA Cyprinid herpesvirus 3 (CyHV-3), more commonly known as koi herpesvirus (KHV), is one of the most virulent viruses of fish. It is a lethally infectious agent that infects common carp and koi (*Cyprinus carpio*) at all stages of their life (Hedrick et al., 2000; Haenen et al., 2004). KHV infections are usually associated with high morbidities and mortalities (up to 95%), resulting in serious environmental damages and severe economic losses (Sunarto et al., 2011; Rakus et al., 2013). This threatening virus had a rapid worldwide spread due to global fish trade and international ornamental koi exhibitions (Gotesman et al., 2013). Classified within the family Alloherpesviridae, genus Cyprinivirus, CyHV-3 is the subject of increasing studies and has become the archetype of alloherpesviruses (Boutier et al., 2015). Despite this “status”, only 19 isolates have been entirely sequenced so far (source: NCBI) since the release of the first complete genome sequences in 2007 (Aoki et al., 2007). Such a low number of full genomes impairs large-scale phylogenomic studies (Gao et al., 2018). On the other hand, KHV infections have been shown to be the result of haplotype mixtures, both in vivo and in vitro (Hammoumi et al., 2016; Klafack et al., 2019). If mixed-haplotype infections probably represent an additional source of diversification for KHV (Renner and Szpara, 2018), they make genomic comparisons more challenging.

KHV has the largest genome among all known herpesviruses, with a size of approximately 295 kb and 156 predicted open reading frames (ORFs) (Aoki et al. 2007). Several studies focusing on the analysis of viral ORFs have shown the implication of some of them in KHV virulence (Boutier et al., 2015; Fuchs et al. 2014). KHV isolates are known to carry mutations in ORFs that are likely to alter gene functions, and these mutations may vary from virus to virus (Gao et al., 2018) and even within viruses (Hammoumi et al., 2016). Aoki et al (2007) hypothesized that virulent KHV would have arisen from a wild-type ancestor by loss of gene function. However, nearly 15 years later, this hypothesis has still not been tested, probably because of the lack of extensive genomic comparisons. SVs may play a key role in this gene function loss, as recently shown by Klafack et al (2019). These authors conducted a comparative study of a cell culture-propagated isolate that suggested that CyHV-3 evolves through an assemblage of haplotypes whose composition changes within cell passages. This study revealed a deletion of 1,363 bp in the ORF150 of the majority of haplotypes after 78 passages (P78), which was not detected after 99 passages. Furthermore, experimental infections showed that the virus passaged 78 times was much less virulent compared to the original wild-type on the one

hand and slightly less virulent compared to the same virus passaged 99 times (P99), highlighting the potentially important role of the ORF150 in the virulence of KHV. Besides, this study demonstrated that haplotype assemblies evolve very rapidly along successive in vitro cell passages during infectious cycles, and raised many questions regarding the mechanisms leading to such rapid gene loss and gain in vitro.

The present study sought to characterize the SV diversity and dynamics in the KHV genome using viruses propagated onto cell cultures. First, P78 and P99 whole virus genomes were sequenced using ultra-deep long-read sequencing, a first with KHV. Then, the obviously pathogenesis-associated ORF150 region (~5 kb) was sequenced in ten intermediate successive cell passages through an Oxford nanopore® amplicon-based sequencing approach to gain insights into the gene loss and gain mechanisms.

Methods

Extraction of high molecular weight DNA from P78 and P99 cell culture passages

The virus isolate used in this study was the same as that previously described in Klafack et al. (2019), i.e. an isolate collected from an infected koi in Taiwan (KHV-T) and passed 99 times onto common carp brain (CCB) cells. Considering previous results, a special focus was made on passages 78 (P78) and 99 (P99). Genomic DNA was extracted from cell cultures stored at -80°C, using the MagAttract HMW DNA Kit (Qiagen). Each frozen culture was thawed quickly in a 37°C water bath, equilibrated to room temperature (25°C) and divided into 12 cell culture aliquots of 250 µL. Tubes were centrifuged at 3,000× g for 1 minute and supernatants were transferred into new 2-mL tubes containing 200 µL of proteinase K and RNase A solution. DNA was subsequently extracted according to the manufacturer's recommendations and eluted in 200 µL distilled water provided in the kit. The 12 replicates of each sample were pooled together and evaporated at room temperature using a vacuum concentrator, to reach a final volume of around 60 µL. Concentrated DNA was quantified by fluorometry (Qubit, ThermoFisher Scientific) and its quality was evaluated by spectrophotometry (Nanodrop 2100) and agarose gel electrophoresis. The final concentration of P78 and P99 was 14.4 and 2.6 ng·µL⁻¹, respectively.

Quantitative PCR assays

Quantitative PCR (qPCR) was applied to evaluate cellular and viral DNA ratio. Two sets of primers were used: primers targeting the ORF150 of CyHV-3 (GenBank #AP008984.1, KHV-J, nt 259,965-260,110: 5'-GAGCGAGGAACTCTACACAAC-3' and 5'-GGTAAGGGTAAAGCAGACCATC-3') and primers targeting the glucokinase gene of *Cyprinus carpio* (GenBank #AF053332.2, nt 225-293: 5'-ACTGCGAGTGGAGACACAT-3' and 5'-TCAGGTGTGGAGGGGACAT-3'). Amplification reactions contained 1 μ L of 2X SYBR Green I Master mix (Roche), 200 nM of each primer, and 1 μ l of template DNA in a final volume of 10 μ L. Amplifications were carried out in a LightCycler 480 (Roche) and cycling conditions consisted of an initial denaturation at 95°C for 5 min followed by 45 cycles of amplification at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 10 sec with a single fluorescence measurement. After amplification, a melting step was applied, which comprised a denaturation at 95°C for 5 sec, a renaturation at 65°C for 60 sec and a heating step from 65 to 97°C with a ramp of 0.1°C per second and a continuous fluorescence acquisition. Specificity of amplification was verified by visual inspection of the melting profiles, and the ratio between cellular and viral DNA was estimated as $2^{-\Delta Cq}$, assuming that each primer pair has an amplification efficiency close to 2 and that each amplicon is present as a single copy per genome.

Genomic library preparation and Oxford Nanopore whole genome sequencing

High-quality genomic DNA from the two samples (P78 and P99) was sequenced using Oxford Nanopore technology®. DNA libraries were prepared using the Ligation Sequencing Kit (SQK-LSK109) according to the manufacturer's instructions (Oxford Nanopore®). A total input amount of 48 μ L (corresponding to 692 ng of P78 and 124 ng of P99 high molecular weight DNA) was used for sequencing library preparation. DNA was first end-repaired using NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End repair, and then cleaned up with Agencourt AMPure XP beads (Beckman Coulter Inc) at a 1:1 bead to DNA ratio. Sixty-one μ L of clean-up elution were transferred into a new 1.5-mL tube for subsequent adapter ligation. Adapter ligation was achieved using NEBNext Quick T4 DNA ligase adapter mix (AMX), ligation buffer (LNB), Long Fragment Buffer (LFB) and Elution Buffer (EB), following the provider's recommendations. The quantity of the retained DNA fragments was measured again by Qubit

fluorometry. The final P78 and P99 DNA amounts were 346 ng and 94 ng, respectively. Each library was directly sequenced on a separate R9.4.1 flow cell using a MinION sequencing device. Sequencing runs were controlled with MinKNOW version 0.49.3.7 and operated for about 30 hours.

Amplicon-based Oxford nanopore sequencing

To specifically investigate ORF150 region, a fragment of ~4.3 kb encompassing the whole ORF150 of CyHV-3 and part of the ORF149 and ORF 151 (nt 257,103-261,345 according to GenBank #AP008984) was sequenced from various intermediate passages of KHV-T (P10, P20, P30, P40, P50, P70, P78, P80, P90 and P99). A ligase-free protocol was used to limit the risk of potential artifacts linked with sample preparation, e.g. the creation of chimeric sequences during the end-repair or ligation steps (White et al., 2017). Genomic DNA was extracted from cell cultures stored at -80°C, using the Nucleospin virus kit (Macherey-Nagel). Purified DNA was subsequently used for PCR amplification with the following primers: 5'-TGGGCGCAATCAAGATGT-3' (F) and 5'-TGAAGTGTCAGGGTCAGAGT-3' (R). PCR was performed with GoTaq G2 DNA Polymerase (Promega) in a final volume of 40 µL containing 1 µL of total genomic DNA, 10 µL of Taq Buffer, 5 µL of dNTPs 2 mM, 2.5 µL of MgCl₂ and 1.25 µL of each primer (10 µM). Cycling conditions were as follows: initial denaturation at 95°C for 10 min, amplification with 40 cycles of 95°C for 10 sec, 60°C for 20 sec, 72°C for 3 min, and final extension at 72°C for 5 min. PCR products were purified using 1X Agencourt AMPure XP beads, tested for purity using the NanoDrop™ One spectrophotometer (ThermoFisher Scientific), and quantified fluorometrically using the Qubit dsDNA High sensitivity kit. DNA libraries were prepared using the Rapid Barcoding kit (SQK-RBK004), following the manufacturer's instructions. For each sample, 400 ng of purified amplicon were adjusted with nuclease-free water to a total volume of 7.5 µL and supplemented with 2.5 µL of Fragmentation Mix RB01-4 (one for each sample). Four barcoded samples were combined with an equimolar ratio by mixing 2.5 µL of each sample in a total volume of 10 µL. Furthermore, as described above, two additional barcodes RB01-2 were used with the passages P78 and P99. Pooled libraries were sequenced on 3 R9.4.1 flow cells (4 barcodes, 4 barcodes, 2 barcodes) for 24 hours and sequencing runs were controlled with MinKNOW version 0.49.3.7.

DNA sequence analysis

For each sample, bases from raw FAST5 files with a pass sequencing tag were recalled using the high-accuracy model of ONT Guppy basecalling software version 4.0.15 which improves the basecalling accuracy. The obtained fastq files were filtered to keep reads with a length ≥ 2 kb, in order to span large structural variants. After the mapping step using minimap2 (Li, 2018), the sequencing depth was calculated for each sample using the plotCoverage tool implemented in deepTools2.0 tool suite (Ramírez et al., 2016). Sequencing coverage was assessed with the bamCoverage tool from the same tool suite and normalized using the RPGC (reads per genome coverage) method (Figure 1).

Structural variant detection

To detect structural variants (SVs) in the P78 and P99 whole genomes, a mapping step followed by BAM filtering was performed. Two aligners were used to map the raw long-reads against the KHV-J AP008984.1 reference genome: minimap2 (Li, 2018) and NGMLR (Sedlazeck et al., 2018). BAM files were then filtered using the option '-F' of Samtools view (Li et al., 2018) with the flag "4" to keep only mapped reads and with the flag "0x800" to remove chimeric reads (inconsistent/supplemental mapping). For P78 and P99, 99.33% and 97.77% of reads were mapped, respectively. Chimeric reads represented 28.05% and 17.74% of the mapped reads in P78 and P99, respectively. The resulting filtered BAM files from each mapper were used as input data for SV caller, Sniffles (Sedlazeck et al., 2018). Only SVs ≥ 30 bp and supported by at least 10 reads were kept in the final VCF files. A cross-validation step was performed using SURVIVOR (Jeffares et al., 2017) by extracting common SVs from each mapper/caller combination for each sample (Figure S1 (Cherif, 2022)). Although the KHV-J reference genome used for the mapping is phylogenetically close to the KHV-T isolate, some genetic diversity exists (Klafack et al. 2017). Hence, a pairwise comparison between P78 and P99 was made to exclude inter-isolate SVs.

The distribution of the different SVs along with P78 and P99 genomes was assessed by estimating their occurrences using a 5 kb sliding window. SNPs and Indels variants were called in P78 and P99 by medaka_variant implemented in medaka (1.4.4) using KHV-J AP008984.1 as a reference genome. To detect structural variants in the amplified region (257,103-261,345)

of P10 to P99 samples, a size filtering step using guppyplex was added to the steps described above. Only reads from 1.5 kb to 8 kb were used for the analysis.

Results

Main features of sequencing data for P78 and P99

A total of 4,900,000 and 2,293,830 long-reads were obtained for P78 and P99, respectively. After filtering, 462,982 long-reads with an average length of 4.96 kb were retained for P78, and 418,034 reads with an average length of 7.06 kb for P99 (Table 1, TableS1 (Cherif, 2022)). 100% of the sampled bases from the P78 genome had at least 5,000 overlapping reads and 100% of the sampled bases from the P99 genome had at least 7,500 overlapping reads (Figure S2 (Cherif, 2022)). The sequencing data covered both P78 and P99 genomes (Figure 1). The ratio between cellular and viral DNA, calculated by qPCR, was 34800 and 33200 for P78 and P99, and the percent of mapped reads 99.33% and 97.77%, respectively.

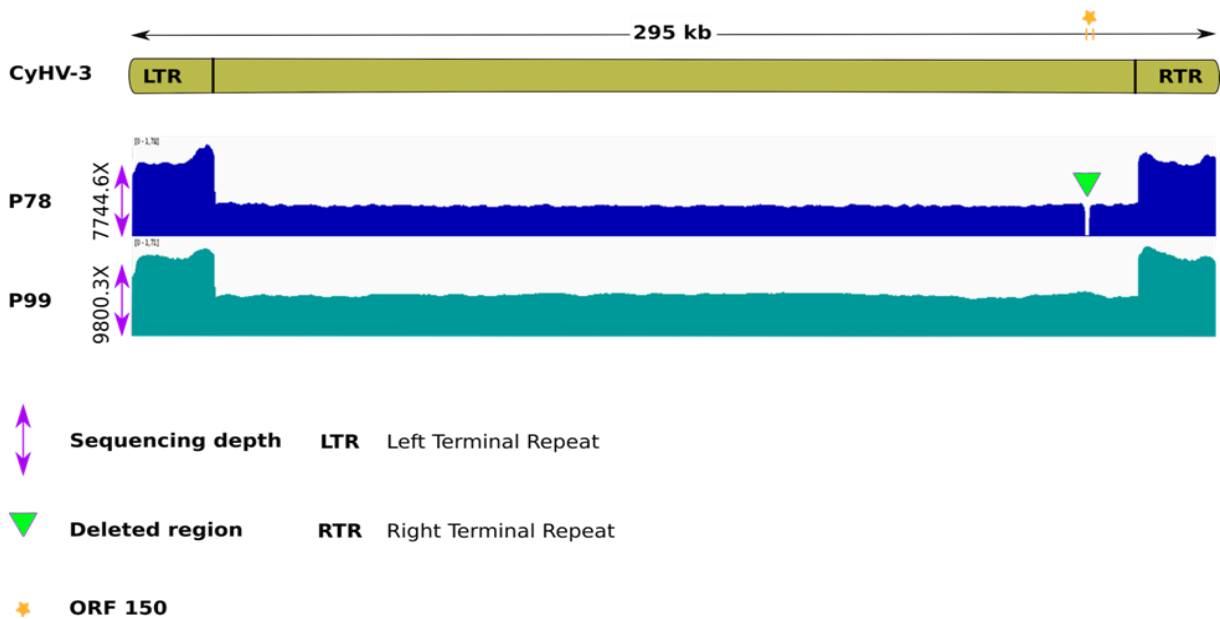


Figure 1: Normalized sequencing coverage for P78 and P99 samples using the RPGC (reads per genome coverage) method. Both P78 and P99 genomes were totally covered by the sequencing. For P78, the coverage break (green triangle) corresponds to the 1.3 kb deletion.

Table 1 Main features of reads obtained for each genome after the read length filtering step. min_len= minimum length, avg_len= average length, max_len= maximum length.

Sample	reads	min_len	avg_len	max_len	N50	Q20(%)	Q30(%)
P78	462982	2000	4960.8	91494	6091	59.53	17.71
P99	418034	2000	7061.4	176512	8845	58.27	19.22

N50= the length for which all sequenced reads of that length or greater sum to 50% of the set's total size
 Q20=1 in 100 probability of an incorrect base call, Q30= 1 in 1000 probability of an incorrect base call.
 Here 59.53% of P78 bases=Q20 and 58.27% of P99 bases=Q20

SV distribution in P78 and P99

For P78, the mapper/caller combination minimap2/Sniffles detected 731 structural variations (SVs), and the combination NGMLR/Sniffles detected 460 SVs (Table S2 (Cherif, 2022)). For P99, the combination minimap2/Sniffles detected 210 SVs and NGMLR/Sniffles detected 397 SVs (Table S2 (Cherif, 2022)). Independently from the mapper/caller combination that was used, P78 showed more SVs than P99 (Table S2 (Cherif, 2022)). After the cross-validation step (extracting common SVs from each mapper/caller combination), 236 and 87 SVs were kept for P78 and P99, respectively. For comparison, the number of small variations (with a size < 30 nt, which were excluded from this study) amounted to 57 and 77 for P78 and P99, respectively.

In both samples, inversions and deletions were the most prevalent SVs. In P78, inversions represented 75% of the events and deletions 20% (Figure 2.A). In P99, 80% of the SVs were inversions and 11% were deletions (Figure 2.A). Inversions were found along the entire genome, with the highest number detected within the [70-75 kb] window in P78 and within the [235-245 kb] and [270-275 kb] windows in P99 (Figure 2.B). In P78, deletions were mainly detected within [10-40 kb], [70-100 kb] and [245-270 kb] windows (Figure 2.B). In P99, the two major deletions were found within [10-20 kb] and [205-215 kb] regions (Figure 2.B). The frequencies of these SVs were low and did not exceed 1% of the total reads (with a few exceptions, Table S3 (Cherif, 2022)). In spite of such low frequencies, it is interesting to note that the most frequent SVs were located in ORFs potentially involved in DNA replication and encapsidation, e.g. ORF33, 46, 47, 55 (Aoki et al, 2007; Table S3 (Cherif, 2022)).

Altogether, these results highlight high SV turnover dynamics during the in vitro infection cycles (from 78 passages to 99) with a clear trend, or bias, towards inversion and deletion events.

Dynamics and impacts of SVs in ORF150 region

Taking advantage of the high-resolution SV detection provided by the long-read sequencing, we looked for the SV events around the potential virulence-linked ORF150 in P78 and P99 (nt 257,103-261,345 according to AP008984.1). Results confirmed that P99 had a reference-like profile with an unmodified ORF150. In P78, the deletion (nt 258,154-259,517; D258153) was found in 6,902 reads (100% of the reads), whereas the reference haplotype was also detected in 30 reads, representing 0.44% of the total 6,734 supporting reads (Figure 3, Tables S4 (Cherif, 2022) (Cherif, 2022)). Surprisingly, 26 reads revealed a haplotype as yet unidentified (INV258153), consisting of an inversion of the same length (1,363 bp) and at the same breakpoints as the deletion. The inverted haplotype (INV258153) in P78 deeply altered the ORF150, by inverting the first 1200 bp of the ORF and 160 bp of the 5'UTR in the middle of the ORF (Figure 3).

In order to trace the unexpected dynamics of gain and loss of the full ORF150 along passages, we searched for the SV turnovers during 10 intermediate passages (P10, P20, P30, P40, P50, P70, P78, P80, P90, P99). This analysis revealed the presence of haplotype D258153 at low frequency (from 0.05 to 0.15% of the reads) in passages P10 to P40 and a strong increase in its frequency at P50 (88.7% of the reads) (Figure 4). The frequency of the haplotype D258153 reached a maximum at P78 (100% of the reads) then dropped quickly at P80 (30.7% of the reads) to stabilize at low frequency (0.31% of readings) at P90., as during the first 40 passages (Figure 4, Table S4 (Cherif, 2022)). Interestingly, shorter deletions of 119 and 881 bp were observed near the 5' end of the ORF150 in P40 and P80, respectively, at low frequencies (0.42 % in P40 and 0.18% in P80) (Figure 4, Table S4 (Cherif, 2022)). The haplotype D258153 completely disappeared at passage 99 (Figure 4, Table S4 (Cherif, 2022)).

This analysis also evidenced several other SVs that alter the structure of ORF150 and of its upstream region, including the beginning of ORF149 (Figure 5). Besides the large deletion, inversions and insertions were also observed in the ORF149-ORF150 region. Inversions were at a low frequency (between 0.01% and 0.53% of the supporting reads) in all

passages except for P70, P90 and P99. P10 and P40 showed the lowest and the highest inversion frequencies, respectively (Figure 5, Table S4 (Cherif, 2022)). A large insertion of about 1 kb appeared in P50 and P70 at moderate frequencies (14,34% and 16.01% of the supporting reads, respectively) to disappear in P78 and re-appear at a lower frequency (6,79% of the supporting reads) in P80. The consensus sequence of this insertion corresponds to the fragment 259,517-260,477 of the KHV genome, with an identity of about 90%. In P90, an intriguing inverted-duplicated haplotype was observed at a low proportion (0.054% of the supporting reads). Surprisingly, P99 exhibited a unique reference-like, SV-free haplotype (Figure 5, Table S4 (Cherif, 2022)). All the variations deeply impacted the structure of ORF150 - and sometimes that of ORF149 as well - by shrinking or increasing its size, causing the ORF149 and ORF150 fusion, inverting the ORF150 sequences and duplicating the ORF150 with the deleted, inserted, inverted and inverted-duplicated haplotypes (Figure 5).

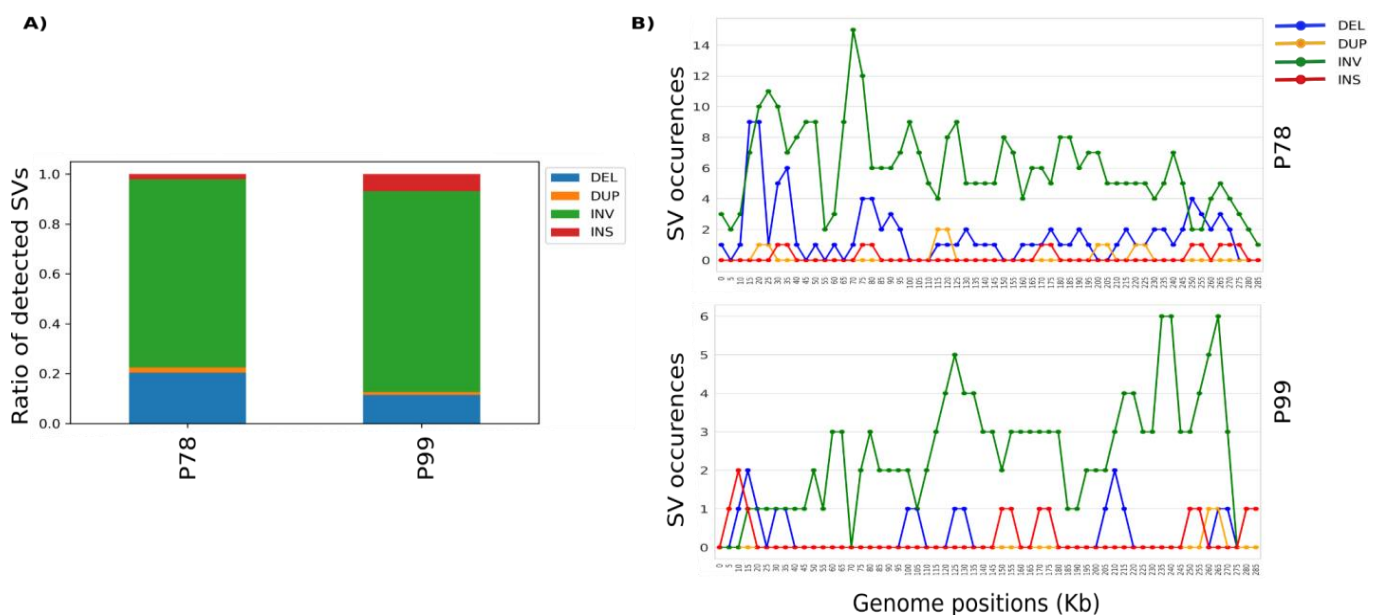


Figure 2: Ratio and distribution of SVs detected in P78 and P99 genomes. **A)** Frequency of each type of SV detected in P78 and P99. **B)** Occurrences and distribution of each SV type in the P78 and P99 genomes using a 5kb window

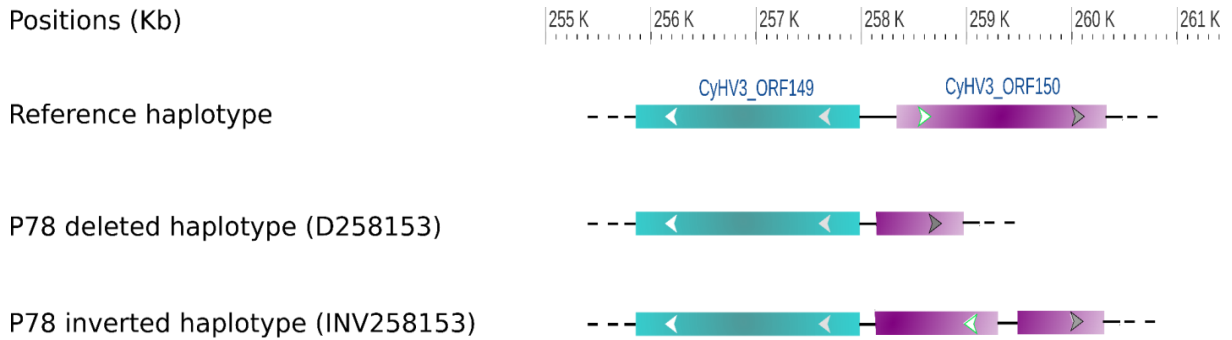


Figure 3: Impact of structural variations D258153 and INV258153 on the P78 genomic structure. The inversion is highlighted by an inverted arrow compared to the reference haplotype

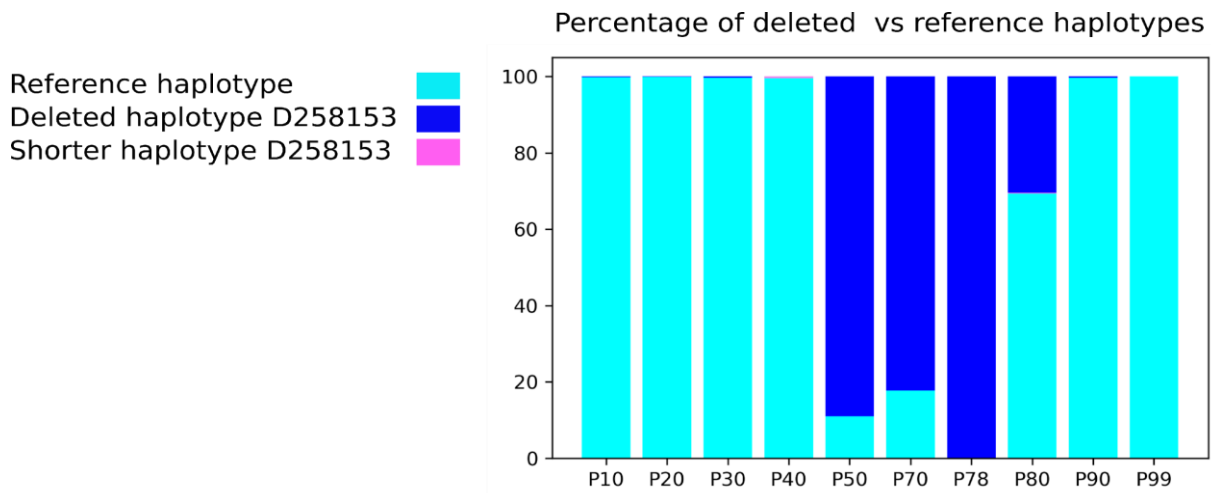


Figure 4: The prevalence of the deleted haplotype during the 10 intermediate passages (P10, P20, P30, P40, P50, P70, P78, P80, P90, P99)

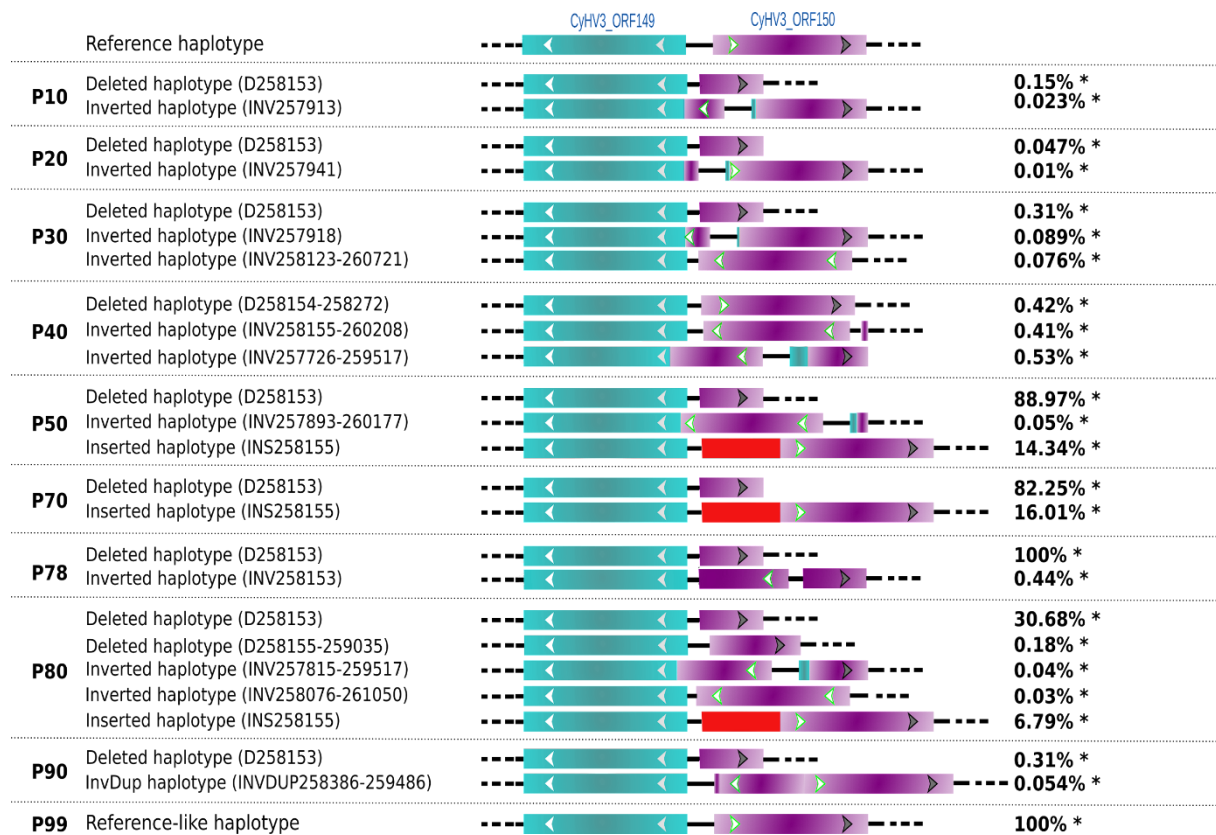


Figure 5: Impact of SV dynamics on the ORF149-ORF150 structure in the successive passages P10, P20, P30, P40, P50, P70, P78, P80, P90 and P99. The inversion is highlighted by an inverted arrow compared to the reference haplotype. Red blocks correspond to an inserted sequence. InvDup = Inverted-duplicated haplotype. *Percentage of reads covering the SVs at given breakpoints.

Discussion

SVs significantly impact the adaptation of viruses to their natural host and environment (Pérez-Losada et al., 2015). Yet the role of SV diversity and dynamics in large DNA viruses is barely known. Ultra-deep long-read sequencing opens unprecedented ways to gain insights into these untapped viral genome polymorphisms. The present study started to tackle the impact of SVs in the evolution of the large dsDNA KHV during cell culture serial passages using ultra-deep whole-genome and amplicon-based sequencing. The sequence data showed a wide

distribution of various SVs along the genome associated with high SV turnover dynamics during the in vitro infection cycles and a clear bias towards inversion and deletion events. Analysis of the pathogenicity-associated ORF150 region in ten serial passages mainly highlighted deletions, inversions and insertions that deeply altered the structure of ORF150.

Serial passages of viruses in cell culture may lead to the accumulation of mutations and gene disruptions (Spatz 2010; Colgrove et al., 2014). These mutations can modify viral adaptation and increase or decrease virulence (Boutier et al., 2017; López-Muñoz et al., 2021; Vancsok et al., 2017). In the case of KHV, a previous work using short-read sequencing showed that 99 consecutive in vitro passages onto CCB cells resulted in the accumulation of less than 60 small variations (<100 nt) (Klafack et al., 2019). It also showed that the haplotype composition can quickly vary along with infection cycles of KHV in vitro. The present study unexpectedly highlighted a high number of structural variations: 87 for P99 and 236 for P78. In contrast, the accumulation of small variations was consistent with what had been observed with short-read sequencing (Klafack et al, 2019). These findings illustrate that long-read sequencing is highly suitable for genome-wide comparisons of viruses. Most importantly, they revealed a hidden source of virus diversification, which had never been reported so far for KHV. They also confirmed that P78 consists of a mixture of undeleted and deleted haplotypes revealing that the undeleted haplotype did not correspond to the native one but to an inverted version of it. We experimentally validated this inversion in P78 by PCR followed by sequencing. Moreover, the inversion found in the middle of the reads excluded the formation of in silico chimeras, i.e., chimeras resulting from the basecaller when two molecules are sequenced in the same pore that undergoes fast reloading (Martin and Legget, 2021). These structural variations form a ‘mosaic’ of viral subpopulations that seem to result from multiple rearrangement events, mainly inversions and deletions and, to a lesser extent, insertions and duplications. Such sub-viral variants often lead to Defective Viral Genomes (DVGs) (Vignuzzi and López 2019). Because of their negative impact on viral replication, some forms of DVGs have been extensively studied, and three pathogenesis-related functions have been well-described: interference with viral replication, immunostimulation, and viral persistence (Marriott and Dimmock 2010; Vignuzzi and López 2019). DVGs play a role in viral production interference by accumulating at higher rates than the full-length viral genomes and consequently interfere with viral replication by taking up the polymerase activity and competing

for structural proteins (Calain and Roux 1995; Portner and Kingsbury 1971). In addition, DVGs can act as primary stimuli and trigger antiviral immunity by inducing the expression of some interleukins and pro-inflammatory cytokines (for review, see Vignuzzi and López 2019; Xiao et al, 2021). However, the implication of DVGs for virus persistence is complex and still unclear. Nevertheless, a combination of viral replication interfering and cycle asynchrony between full-length viruses and DVGs seems to establish chronic infection (for review, see Vignuzzi and López 2019).

The observed dynamics of structural variations in the KHV genomes during cell culture passages where selection pressures are virtually non-existent may indicate an accumulation of DVGs impacting the pathogenicity. Indeed, Klafack et al. (2019) showed that serial passages significantly attenuated this infectious virus. This attenuation was associated with a truncated KHV genome bearing a 1.3-kb deletion that removes the majority of the ORF150. A very recent study showed that partial or complete depletion of ORF150 leads to a clear attenuation of the virus. It also brought in vivo evidence that ORF150 may down-regulate the inflammatory response of carp to enhance viral proliferation, thus confirming a key role of ORF150 product in KHV virulence (Klafack et al., 2022 submitted). ORF150 encodes a protein with a Really Interesting New Gene (RING) domain that is predicted to span 628 amino acids (Li et al, 2015; Aoki et al, 2007). This RING domain contains the HC (C3HC4) type of RING structure, which is involved in the ubiquitination pathway, by acting as an E3 ubiquitin-protein ligase (He and Kwang 2008). Viruses that encode RING finger like-ubiquitin ligases (E3s) may evade host immune responses and also hijack the host's RING E3 to enhance their replication (Zhang et al., 2018). In aquatic viruses, RING family genes have been reported to be involved in virus latency, replication, and host protein degradation (Shekar and Venugopal 2019; Wang et al., 2021).

As previously observed, the most significant difference affecting all viral haplotypes between P78 and P99 was around the ORF150. For this reason, we focused on this interesting region to determine the passage number at which the presence of the deletion inversion first arose, by sequencing selected representative passages (P10, P20, P30, P40, P50, P70, P80 and P90). We found that deleted and inverted haplotypes appeared as soon as P10 and that their proportion varied along the successive passages. The most striking feature was the rapid and

total disappearance of the deletion between P78 and P99, raising many questions regarding the mechanisms that led to the clearance of this major haplotype.

The rapid SV turnover of DNA viruses, including herpesviruses, likely involves recombination (Szpara and VanDoorslaer, 2021; Renner and Szpara, 2018; Cudini et al., 2019; Wilkinson and Weller, 2003; Kolb et al., 2017; Tomer et al., 2019), which is often linked to replication and DNA repair, as well as errors during viral genome replication (Kulkarni and Fortunato 2011; Xiaofei and Kowalik 2014). In the present case, the good conservation of breakpoints around ORF150 may signify homologous recombination. However, whether this recombination occurs within the same genomic entities or between different viruses remains open. It would be interesting to assess the involvement of each of these mechanisms in generating the observed structural diversity. The multiple mechanisms of DNA virus evolution beyond single nucleotide substitutions likely confer KHV a high level of evolutionary adaptability.

Classically, the generation of live-attenuated vaccines is achieved by passaging the virus in cell culture under different conditions (in different host species or at lower or higher temperatures), in order to induce mutation accumulation that supports viral adaptation to the specific conditions and provides viral attenuation (Minor, 2015, Hanley 2011). With the exception of the OPV polio vaccine viruses (Kew et al., 2005), the exact mechanisms by which these mutations lead to attenuated phenotypes are usually poorly characterized (Lauring et al., 2010). However, live-attenuated viruses can revert to virulent phenotypes either by reversions (as shown here between P78 and P99), the introduction of compensatory mutations, or recombination with viruses belonging to the same genus (Cann et al., 1984; Bull et al., 2018; Muslin et al., 2019). Additionally, the combination of multiple live-attenuated viruses may result in competition or facilitation between individual vaccine viruses, resulting in undesirable increases in virulence or decreases in immunogenicity (Hanley 2011; Pereira-Gomez et al., 2021). Recently, genetic engineering has led to many novel approaches to generating live-attenuated virus vaccines containing modifications to prevent virulence reversion (Yeh et al., 2020) and improve interferences among multiple vaccine strains (Pereira-Gomez et al., 2021).

Conclusion

Our findings confirm that CyHV-3 can evolve rapidly during infectious cycles in cell culture, and SVs are a major component in the evolutionary process of this virus. SVs are extremely dynamic under in vitro controlled conditions, and it would now be interesting to evaluate their dynamics in vivo. The present study also contributes to the basic research on the mechanisms underlying attenuation and may have important outcomes for the design of safe live-attenuated vaccine formulations.

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Data, scripts and codes availability

Raw sequences (fastq files) were stored in the public Sequence Read Archive (SRA) repository and can be accessed under the bioproject PRJNA511566. The original genetic data yielded by genotyping (in vcf format), codes and additional metadata are available in a publicly-available OSF repository: <https://doi.org/10.17605/osf.io/3c2ag>.

Supplementary material

Supplementary material is available online: <https://doi.org/10.17605/osf.io/3c2ag>

Conflict of interest disclosure

The authors of this preprint declare that they have no conflict of interest with the content of this article.

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4. Chapter 2 - Prevalence of Cyprinid herpesvirus 3 and ORF150 genomic variations in carp populations of Indonesia

Context

CyHV-3 can undergo rapid evolution during its infectious cycles in cell culture, which is characterised by the absence of selection pressure, such as the immune system. In the previous chapter, it was shown that structural variations play a crucial role in the virus's evolutionary process *in vitro*. These findings led to the question of whether some of these dynamic structural variations also occur *in vivo* and contribute to the modulation of CyHV-3 pathogenicity. Considering the role of ORF150 in CyHV-3 virulence, and the difficulty to carry out genome-wide analyses from *in vivo* samples, a strategy combining qPCR and amplicon-based sequencing was applied on ORF150 region, which was shown, in the previous chapter, to be highly dynamic in terms of structural variations. We investigated common carp farms in Indonesia, from areas with different levels of endemicity. This study therefore provided an opportunity to estimate the prevalence of CyHV-3 in one of the largest carp-breeding countries, Indonesia.

RESEARCH ARTICLE

Prevalence of Cyprinid herpesvirus 3 and ORF150 genomic variations in carp populations of Indonesia

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Cyprinid herpesvirus-3 (CyHV-3) is the etiological agent of koi herpesvirus disease (KHVD) in common and koi carp farming. This highly contagious pathogen has a 295-kb genome that harbours 156 open reading frames. Recent *in vitro* experimental evolution studies highlighted strong dynamics of genomic structural variations (SVs), in particular in the region of ORF150, an ORF potentially involved in virus multiplication and host inflammatory response. Among these SVs, a 1363-bp deletion could be associated with a loss of virulence. The present study aimed at investigating the genomic variations in the ORF150 region, and especially the deletion, in viruses isolated from carp populations of Indonesia. A screening of 236 fish from 43 different farms revealed a high prevalence of CyHV-3 (nearly 70%), both in symptomatic and asymptomatic common carp. However, in contrast with the results obtained *in vitro*, long read sequencing of the ORF150 region revealed a low level of genetic variations and the absence of the 1363-pb deletion. The complex interactions between the virus, the environment and the host, particularly the immune system, probably play an important role in this reduced variability.

Introduction

Cyprinid herpesvirus 3 (CyHV-3), recently renamed *Cyvirius cyprinidallo3* (ICTV 2022), and also known as Koi Herpesvirus, is a highly pathogenic virus responsible for the Koi Herpesvirus Disease (KHVD) in common carp and ornamental koi *Cyprinus carpio*. CyHV-3 belongs to the genus *Cyvirius*, family *Alloherpesviridae* and order *Herpesvirales* (Waltzek et al.

2005). CyHV-3 causes massive mortalities in common carp farming after 5–7 days and has been highlighted as a serious threat to aquaculture and wild populations (Boutier et al. 2015). Since its emergence in 1996, CyHV-3 is present in almost all continents (Rakus et al. 2013). Several factors, such as virus virulence, association with bacterial infections, host condition and water temperature, have been shown to determine the outcome of CyHV-3 infection (Omori and Adams 2011; Padhi et al. 2019; Yi et al. 2014). In most farming settings, preventive efforts against CyHV-3 infection rely on avoiding exposure to the disease by applying efficient biosecurity measures as well as surveillance programmes (Amin et al. 2018).

CyHV-3 is a structurally complex virus possessing the largest genome (295 kb) known among *alloherpesviruses*, which encodes 156 putative genes, or open reading frames (ORFs) (Aoki et al. 2007). The large size of its genome and the restricted number of full-length sequences obtained from *in vivo* samples has become an obstacle to understanding its evolution. Using specific genetic markers, Sunarto et al. (2011) hypothesized that Indonesian isolates all originate from a single introduction, followed by a rapid spread over the entire country.

Several ORFs have been investigated for their specific role in the virus biology, especially its pathogenicity (Liu et al. 2020 ; Gao et al. 2023). This is the case of ORF150, which belongs to the really interesting new genes (RING), potentially involved in virus-host interaction by hijacking the cellular E3 ligases (Heilingloh et al. 2014). CyHV-3 ORF150 may down-regulate carp's inflammatory response to enhance viral proliferation (Klafack et al. 2022). Of interest, a deletion of 1363 bp in ORF150 was shown to be associated with a loss of virulence during challenge tests and served for the elaboration of a vaccine candidate (Klafack et al. 2022). Genomic comparisons of the same cell culture-propagated isolate revealed a dynamic accumulation of structural variations in ORF150 along successive passages on CCB cells (Fuandila et al. 2022). These comparisons showed that CyHV-3 can evolve rapidly during infectious cycles in cell culture (without selection pressure) and that structural variations are an essential component in the evolutionary process of this virus. These results raised the question of whether such structural variations, particularly the deletion in ORF150, also occur *in vivo* to modulate the virus pathogenicity. To address this question, 43 common carp farms in Indonesia's enzootic and non-enzootic areas were investigated. Both qPCR-based and sequencing analyses were used to detect the presence of this deleted variant in the associated CyHV-3 strains and

identify other potential structural variations in the ORF150. This study was also an opportunity to evaluate the prevalence of CyHV-3 in Indonesia's biggest carp production areas.

Materials and Methods

Field sample collection

Sample collection of common carp (*C. carpio*) was carried out between January and May 2022 in 43 farms distributed in 7 provinces throughout Java and Sumatra islands ([Figure 1](#)). Fish weighing 10–500 g were harvested from the ponds, and a small piece of gill was taken out, placed in 95% ethanol and kept at -20°C upon arrival to the lab. Fish were then released back into their pond. Information on the collection date, area, size of the fish and number of fish collected at each site are gathered in Supplementary Table 1. In the present study, 37 fish pond farms under various ownerships, and six floating net cage farms, were collected. Sampling farms corresponded to both enzootic and non-enzootic areas of Indonesia (according to fish farmers), and sampling occurred during both epidemic and non-epidemic periods (Table S1). Fish were classified into three groups based on the level of observed clinical signs (Monaghan et al. 2014): 1 (severe clinical signs), 2 (intermediate clinical signs) and 3 (absence of clinical signs). A total of 236 gill samples were collected. Water parameters such as dissolved oxygen, temperature and pH were also

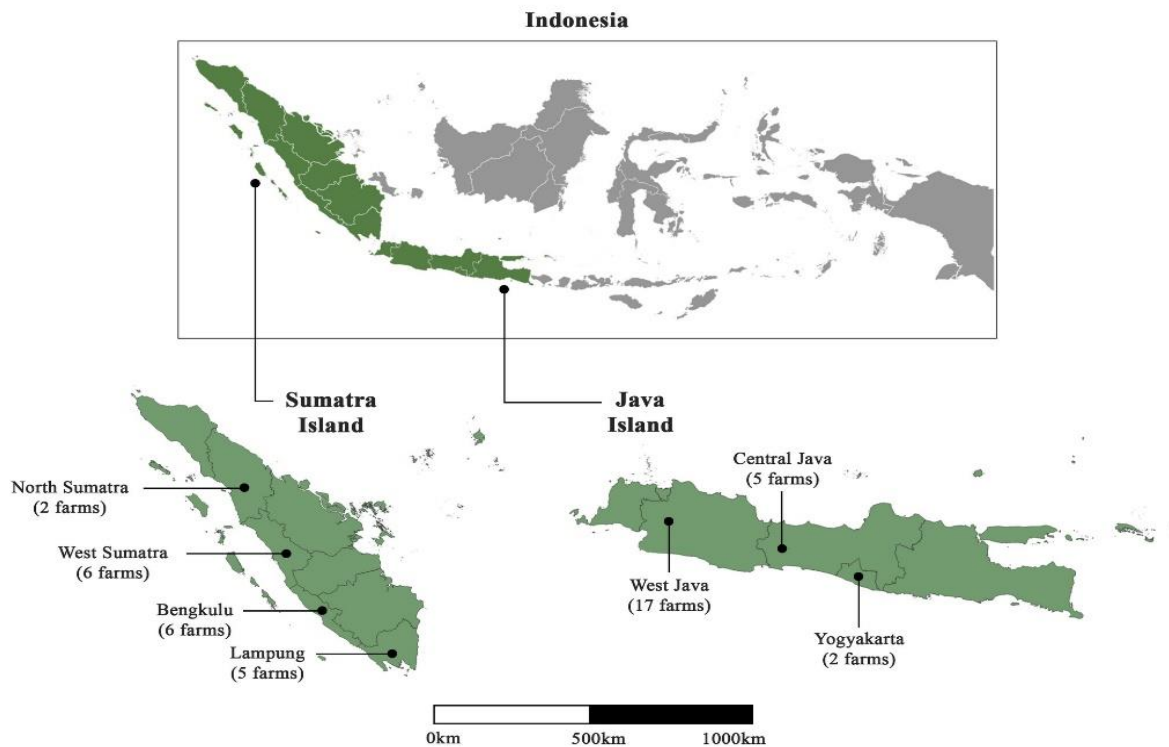


Figure 1. Locations of carp farms sampled in Indonesia

DNA Extraction

Viral genomic DNA was extracted using the Nucleospin virus extraction kit (Macherey Nagel, Germany), following the manufacturer's instructions. Briefly, 10-15 mg of gills were grounded using a pestle in a 1.5 mL microtube, and then tissue homogenates were centrifuged at 4000 x g for 10 min at 25°C. Supernatants were transferred into a new 1.5 mL tube to add proteinase K and RNase A solutions. After several wash steps, DNA was eluted with 60 µL of distilled water provided in the kit. Purity of the obtained DNA was verified using a Nanodrop spectrophotometer.

qPCR assays

All 236 samples were tested for i) CyHV-3 carriage and ii) the potential presence of the 1.3-kb deletion in ORF150 (Klafack et al. 2019). For CyHV-3 detection, the improved *Sph* I-5 primers (Yuasa et al. 2005) were used, and DNA was amplified exactly as described in Avarre et al. (2012). Each sample was run in triplicate, and each run contained several no-

template controls. Samples were classified into three categories: + when the three replicates had a cycle of quantification (Cq) lower than 30 and a single melting peak; +/- when only one or two replicates fulfilled the above criteria; - when the three replicates were negative (Cq >30). To evaluate the presence of the deletion in the Indonesian viral strains, two primer sets were used, as described in Klafack et al. (2019): one set targeting the ORF150 of CyHV-3 (nt 259,965 – 260,110 according to GenBank #[AP008984.1](#)), and the other located in the putative deletion (nt 258,548 – 258,659 according to GenBank #[AP008984.1](#)). Primer sequences are displayed in [Table 1](#). Amplification reactions contained 2.5 µL of 2X SYBR Green I Master mix (Roche), 200 nM of each primer, and 0.5 µL of template DNA in a final volume of 5 µL. qPCR amplifications were carried out in a LightCycler® 480 (Roche). Cycling conditions consisted of an initial denaturation at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 10 sec with a single fluorescence measurement. A melting curve was applied to verify the specificity of the amplified products. It consisted of a denaturation at 95 °C for 5 sec, a renaturation at 65 °C for 60 sec and a heating step from 65–97 °C with a ramp of 0.1 °C per second and a continuous fluorescence acquisition. As tested on cell-culture propagated CyHV-3 strains (not shown), the deletion can be confidently detected under two conditions: the ratio between Cq of the two primer sets is significantly lower than one, and the melting profiles of both amplicons are pure.

Table 1. List of primer sets used for detecting CyHV-3 by qPCR and conventional PCR (cPCR)

Name	Primer sequence (5'–3')	Reference
qPCR		
SphI-5-F	GACACATGTTACAATGGTCGC	Yuasa et al. 2005
SphI-5-R	CAACTGCAACATACCGTCAAG	
ORF150-131F	GCTGGACCTGTCACAATTCTAT	Klafack et al. 2019
ORF150-243R	TGGTCCAGTAGACGGTTGA	
ORF1501589F	TGAGGATGCAGAAGCAGTGGATGT	Klafack et al. 2019
ORF1501694R	GGTAAGGGTAAAGCAGACCATC	
cPCR		
ORF150-F	TGGGCGCAATCAAGATGT	Fuandila et al. 2022
ORF150-R	CCCATAACCAGCATACCATGTC	

Amplicon-based MinIon sequencing

Five positive CyHV-3 samples presenting a high viral load (attested by low Cq values) were subjected to an amplicon-based sequencing. To specifically analyse the whole ORF150 genomic region of CyHV-3 (nt 257,103-260,484 according to GenBank #AP008984), a fragment of ~3.3 kb encompassing the whole ORF150 was amplified with a specifically designed primer pair ([Table 1](#)). PCR was performed with GoTaq G2 DNA Polymerase (Promega) in a final volume of 40 μ L containing 1 μ L of total genomic DNA, 10 μ L of Taq buffer, 5 μ L of 2-mM dNTPs, 2.5 μ L of MgCl₂ and 1.25 μ L of each primer (10 μ M). Cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec, 72°C for 3 min, and final extension at 72°C for 5 min. Two rounds of PCR using the same primer pair were necessary to obtain an amount of amplicons sufficient for sequencing. The size of the amplicons was verified by gel electrophoresis with 1% (w/v) agarose (Dutscher Scientific) in Tris-acetate EDTA buffer.

Single amplicon bands of the expected size (3.3 kb) were purified using 1X Agencourt AMPure XP beads. The purity of the obtained DNA was tested using the NanoDrop™ One spectrophotometer (ThermoFisher Scientific). The quantity of DNA was estimated using the Qubit dsDNA High sensitivity kit. DNA libraries were prepared using the Rapid Barcoding kit (SQKRBK004, Oxford Nanopore Technologies), following the manufacturer's instructions. For each sample, 400 ng of purified amplicon were adjusted with nuclease-free water to a total volume of 7.5 μ L and supplemented with 2.5 μ L of Fragmentation Mix RB01-5 (one for each sample). The five barcoded samples were combined in an equimolar ratio and the pooled libraries were sequenced on an R9.4.1 flow cell, using MinKNOW version 0.49.3.7. Raw fast5 files were recalled using the high-accuracy model of ONT Guppy basecalling software version 6.1.5, and the resulting fastq files were analysed exactly as described in Fuandila et al. (2022). The resulting filtered BAM files (Sedlazeck et al. 2018). Sequencing depth was calculated for each sample using the plotCoverage tool implemented in deepTools2.0 tool suite (Ramírez et al. 2016).

Results and Discussion

qPCR analysis using *Sph* I-5 primers revealed that 165 of 236 sampled fish were positive for CyHV-3. Surprisingly, only 66 fish presented obvious severe clinical signs ([Table 2](#)). All

of the CyHV-3-negative fish looked healthy. A high number of KHVD-negative fish were found in West Sumatra. Though farms from this region had never experienced CyHV-3 infections, several samples were found positive for the virus. All carp from South Bengkulu, Central Java and Yogyakarta farms looked asymptomatic, yet most tested positive for CyHV-3. Even though the situation was not epidemic during sampling, these areas have experienced CyHV-3 outbreaks in recent years. Some farms from Central Java and Lampung showed both CyHV-3-negative and positive (with a single melting peak) fish. The negative CyHV-3 results can probably be explained by a low level of infection, as already observed (Monaghan et al. 2015; Jin et al. 2020). In contrast, farms from Lampung Bay showed variable levels of CyHV-3 infection, even between very close farms. According to the farmers, this region has frequently experienced CyHV-3 outbreaks. Most of the CyHV-3-positive fish were devoid of clinical signs and had previously experienced KHVD. It is known that common carp exposed to CyHV-3 can become persistently infected with the virus (St-Hilaire et al. 2005).

The vast majority of samples from West Java were associated with severe symptoms and most of them presented a high level of amplification. West Java is known to be enzootic for CyHV-3 and has been reported to experience common carp mortalities up to 100% (Sunarto, Rukyani, et al. 2005; Avarre et al. 2012). Several locations were facing an outbreak onset when sampling occurred. Since abiotic factors are known to influence the outcome of a viral infection (Fabian et al. 2016), water temperature, dissolved oxygen and pH were recorded in most sampled farms. Overall, these water parameters were adequate for carp growth in all systems (Oyugi et al. 2012). Permissive temperatures are generally reported to range from 18 °C to 28°C (Omori and Adams 2011; Fabian et al. 2015). However, the present study showed that water temperatures in common carp farming frequently exceeded this permissive temperature range, between 28 °C and 31 °C. A previous report highlighted that such high temperatures in Indonesia did not prevent the outbreaks, though they could also contribute to maintaining a high level of asymptomatic carriers (Avarre et al. 2012). Altogether, these results show that CyHV-3 is still highly prevalent across Java and Sumatra islands, regions where the virus spread quickly after its first introduction to Indonesia (Sunarto et al. 2011).

Table 2. Detection of CyHV-3 by qPCR in common carp isolated in Indonesia

Province	Region	Farms	Samples	qPCR (+)	qPCR (±)	qPCR (-)
West Sumatra	Pasaman	PU	4		3	1
		PSM	5		2	3
	Lima Puluh Kota	KT50	10		2	8
		BP-1	5	1		4
	Solok	BP-2	5		1	4
BP-3		5			5	
Bengkulu	South Bengkulu	KS	3		3	
		KE	3		3	
		SN	2	1	1	
	North Bengkulu	BG	3		1	2
		ZAM	2			2
North Sumatra	Sibolga	PA	2			2
		SBL	5		5	
	Padang Sidempuan	BPS	5		3	2
Lampung	West Lampung	LB	5	5		
		LWT	5		3	2
	North Lampung	LTJ	10	2	7	1
		LPP	10		6	4
	Pringsewu	LPF	10		2	8
Central Java		Magelang	MGB	4		2
	MGP		5		4	1
	Wonosobo	MTI	3	1	2	
		WO	6		4	2
	Ambarawa	AMB	5		3	2
West Java	Sukabumi	JD	3	3		
		PLP	3		1	2
		NS	5		3	2
		SKBM-P	5		4	1
		SKBB	10		10	
	Garut	PLT	5		1	4
		GT	5	2	3	
		GR	3		2	1
	Subang	SBG-H	3	3		
		SBG-KFMN	5	5		
		SBG-O	3	1	1	1
	Cianjur	SBG	15	12	3	
		CRT	5	5		
	Bogor	E	10	1	5	4
		BE	5	5		
Purwakarta	JTR	7	5	2		
	JTR-F	7	4	3		
Yogyakarta	Cangkringan	BC	10	6	4	
		NO	5	4		1
Total		43	236	66	99	71

The potential presence of the deletion located in ORF150 (ORF150-del) was investigated in all of the CyHV-3-positive samples. No notable amplification differences were observed between the two primer pairs, suggesting that the ORF150-del haplotype was not present in any sample from Indonesia. To confirm this finding, deep sequencing was realised on the samples that presented the highest viral loads, using the MinIon technology. A sufficient amount of DNA could be obtained for five of them. These samples originated from different farms in West Java: BE-1, BE-2, CRT-1, CRT-2 and DJ-6. The average sequencing depth for the five samples generally ranged from 8,000X to 20,000X. Analysis of the obtained 3.3-kb region revealed a limited number of structural variations (inversions, deletions, duplications, and insertions with a length > 30 nucleotides), all of which supported by a low number of reads (between 11 and 110). This analysis confirmed the absence of the large 1.3-kb deletion in ORF150. In addition, six single nucleotide polymorphisms (SNPs) were recorded in this region with a frequency higher than 5%: five were samplespecific, and one was shared by all samples (Supplementary Table 2). All these SNPs were located within ORF150, and four of them were silent (i.e. had no consequence on ORF150 sequence). The SNP shared by all samples resulted in the change of an arginine for a histidine, with a frequency above 90%. Considering that these two amino acids have the same biochemical properties (they are both basic), it is unlikely that this modification has significant consequences on the protein structure. The last one had a frequency of 23 % and only concerned sample CRT-2. Altogether, these results reveal a low level of variations in this region. This contrasts with what was observed in *in vitro* samples (Klafack et al. 2019; Fuandila et al. 2022) and suggests that the genomic plasticity of CyHV-3 is much lower *in vivo* than *in vitro* (Hammoumi et al. 2016). Genomic variations that are extremely dynamic under *in vitro* controlled conditions are probably constrained *in vivo* where environmental conditions and the host immune response represent strong selection pressures. The absence of the deleted form of ORF150 tends to indicate that this SV is not, at least alone, involved in the modulation of CyHV-3 pathogenicity *in vivo*. However, one cannot exclude that this deletion was not found in asymptomatic fish because of low viral loads, which renders detection challenging. Consequently, further confirmation on a larger set of fish is required. Though still technically difficult because of the high content of host DNA in gill samples, long-read sequencing of the entire viral genome would enable study of SVs along the entire length

of the viral genome and identify other regions subject to selection and potentially involved in CyHV-3 virulence

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supplementary materials

Supplementary Table 2

Download: <https://eafpbulletin.scholasticahq.com/article/84009-prevalence-of-cyprinidherpesvirus-3-and-orf150-genomic-variations-in-carp-populations-of-indonesia/attachment/171653.docx>

Supplementary Table 1

Download: <https://eafpbulletin.scholasticahq.com/article/84009-prevalence-of-cyprinid-herpesvirus-3-and-orf150-genomic-variations-in-carp-populations-of-indonesia/attachment/171654.xlsx>

5. Chapter 3 – Effect of *in vitro* serial passages and thermal stress on Cyprinid herpesvirus 3 evolution

Context

The results obtained in chapter 1 showed that SVs may play a key role in the *in vitro* evolution of CyHV-3. However, we don't know to what extent haplotype assemblies are regulated by a random process or influenced by selective pressures. To begin addressing this question, an isolate of CyHV-3, obtained during an outbreak in Italy, was propagated in 10 identical subcultures of CCB cells. The viral populations of each subculture were independently and serially passaged 50 times under identical conditions. Since temperature has a great effect on the outcome of CyHV-3 infections, a thermal stress was artificially applied at passage 26, where five sub-cultures were exposed to 15°C and the five others to 28°C. Using whole-genome sequencing, we evaluated the nature and frequencies of variations, including SNPs and SVs, which accumulated in the subcultures before and after this thermal stress.

Effect of *in vitro* serial passages and thermal stress on *Cyprinid herpesvirus 3* evolution

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Abstract

The importance of structural variations in the genetic diversity of viruses is still largely unknown. To tackle this question, we studied the *in vitro* evolution of the large DNA *Cyprinid herpesvirus 3* (CyHV-3), a highly pathogenic virus for common and koi carp. An Italian isolate of CyHV-3 was propagated in 10 identical subcultures and serially passaged 50 times under identical experimental conditions. At passage 26 (P26), a thermal stress was applied to evaluate its effect on the accumulation of variations. Long-read sequencing of all subcultures from P15, P30 and P50 was realised, and both single nucleotide polymorphisms (SNPs) and structural variations (SVs) were searched against the KHV-U reference genome. Analysis of genome-wide sequences revealed that SNPs accumulated steadily after successive passages, with a uniform distribution along the genome. The thermal stress had apparently no effect on their accumulation, and even fixation, in the viral population. The accumulation of SVs was slower, and followed a different trend: their number was relatively low at P15, then decreased at P30 to significantly increase again at P50. Moreover, most of the SVs recorded at P15 were not detected at P30 and P50, suggesting that the thermal stress affected their dynamics of accumulation. Interestingly, CyHV-3 showed stronger and earlier cytopathic effects from P38 onwards, suggesting an adaptation to the cell cultures. This observation was concomitant with the fixation in the viral genomes of all subcultures of a specific SNP in ORF131, which was previously demonstrated to be involved in an increased *in vitro* fitness of CyHV-3. Finally, contrary to what could be expected from former studies, no deletion was detected in ORF150, an ORF associated with CyHV-3 virulence. This study contributes to our knowledge on the mechanisms of viral genetic diversity, which is essential for developing preventive strategies such as vaccines.

Introduction

When available, cell cultures offer great possibilities for studying the molecular bases of viral pathogenicity and viral evolution. *Cyprinid herpesvirus 3* (CyHV-3) is an enveloped icosahedral virus packaging a 295-kb double-stranded linear DNA genome. CyHV-3 is the etiological agent of an emerging and mortal disease in common and koi carp. CyHV-3 has been propagated *in vitro* using a series of common carp cell cultures, including koi fin (KF) caudal cells or common carp brain (CCB) cells (Rahmati-Holasoo et al. 2016; Bergmann et al. 2020). However, propagation in cell culture was not always successful. For instance, it took more than four years to obtain the first Indonesian CyHV-3 isolate in cell cultures (Sunarto et al., 2011). The same difficulties were reported in other geographic areas, such as South Korea (Gomez et al, 2011), Japan (Sano et al., 2004) or Thailand (Pikulkaew et al.m 2009). This is probably one of the reasons why the number of CyHV-3 genome-wide studies is still very limited. Another reason is the difficulty to obtain enough viral DNA from infected animals to recover its full genome. Even though a strategy for specific CyHV-3 genome enrichment was successfully applied on several fish samples, analysis of the obtained sequences revealed the cohabitation of many haplotypes, rendering impossible the reconstruction of complete genomes (Hammoui et al., 2016). The same phenomenon was also observed for viral samples isolated in cell cultures, in which haplotype proportions could quickly vary from one passage to another (Klafack et al., 2019). Additionally, it has been demonstrated that once a CyHV-3 viral isolate is established in cell culture, it experiences adaptation that may alter its behaviour (Boutier et al. 2015;). Such *in vitro* systems are indeed generally devoid of selective pressures, such as the immune system, and genomic variations may accumulate without any specific selection (Cheng et al., 2010). Therefore, if cell culture is probably not the ideal system to study the evolution of CyHV-3, it can yet inform on the mechanisms that dictate its diversification. Besides, it is also an appreciable way to make investigations with respect to animal welfare, as it does not need to sacrifice any animal.

Single nucleotide polymorphisms (SNPs) have long been considered the major source of genetic diversity in living organisms. The development of tools for genome-wide studies, and especially the third generation of sequencing technologies, has recently enlightened the role of structural variations (SVs) in species diversification (Wellenreuther et al., 2019). However, the contribution of SVs in the evolution of viral genomes is still poorly investigated, especially

at the genome scale (Mahiet et al., 2012). It was recently shown that structural variations may play a key role in the *in vitro* evolution of CyHV-3 (Fuandila et al., 2022). However, the factors potentially associated with this phenomenon could not be determined, such as, for example, potential environmental disturbances.

To begin addressing this question, a CyHV-3 strain isolated in Italy was propagated in 10 identical subcultures of CCB cells. The viral populations isolated from each subculture were independently and serially passaged 50 times under the same conditions. It is known that the outcome of CyHV-3 infections is influenced by temperature, both *in vitro* and *in vivo*. CyHV-3 replication and disease progression are strongly correlated with water temperature, with the disease occurring mainly at water temperatures between 18 and 28°C (Boutier et al., 2015 ; Yuasa, 2005). The optimal temperature range for the replication of this virus is 16–23°C (Yuasa et al., 2008). Cool temperatures inhibit CyHV-3 transmission due to slow virus replication, whereas virus propagation and gene transcription are gradually turned off when cells move from permissive to non-permissive temperatures above 28°C (Dishon et al. 2007; Ilouze 2012). For this reason, an environmental stress was artificially introduced at passage 26. Five sub-cultures were exposed to 15°C and the five others to 28°C, for 24 hours. Using whole-genome sequencing, we evaluated the nature and frequencies of variations in subcultures of three passages: P15 (before thermal stress), P30 and P50 (after thermal stress). A comparison of these passages indicates that, if structural variations accumulated quickly, they were however more susceptible to the thermal stress than single nucleotide polymorphisms.

MATERIALS AND METHODS

CyHV-3 propagation onto common carp brain (CCB) cells

The strain of CyHV-3 used in this study (n°197/ITT-V12) was kindly provided by the Istituto Zooprofilattico delle Venezie (Italy). It was isolated in 2012 from imported koi suffering a mass mortality outbreak in Italy (Pretto et al., 2013). CCB cells were cultured in Glasgow minimum essential medium (GMEM) supplemented with 10 % fetal calf serum (FCS), 10 % tryptose phosphate and 1% antibiotic (Kanamicyn, penicillin) and maintained at 24°C. After reaching 90% of confluence, cells were detached from the initial flask using 0.5 mL of trypsin and were split into 12.5-cm² flasks containing fresh medium at a density of 2.5×10⁵ CCB cells/mL. After a 24-hour incubation at 24°C, cells were inoculated with 0.5 mL of CyHV-3

suspension and were incubated for 1.5 h at 24°C for virus adsorption. Viral inocula were removed, and flasks were filled with 4 mL of fresh medium. Infected cells were incubated at 24°C until cytopathic effects (CPE) covered about 80% of the cell monolayers, which was generally achieved in seven days. Infected cells were harvested by scraping and removing cell debris by centrifugation at 4000 g for 10 minutes. Supernatants containing the virus were transferred into aliquots that served to infect the next cell culture; the remainder of the viral suspension was stored at -80°C for future use. Cells were observed daily, and commonly infected cells started to lose their adherence to the flask surface at about 6 dpi. Each cycle of infection and harvest was considered as a passage.

Experimental design

To investigate the evolution of CyHV-3 in individual cultures, ten flasks of 24-hour cells were inoculated with the same viral suspension, representing ten clonal subcultures of the initial isolate (Figure 1). The ten subcultures were strictly maintained under the same conditions and serially passaged 50 times. In order to increase the viral titer of the provided viral suspension, the strain was first passaged 3 times on CCB cells before start the experiment. Therefore, the first infection of the present experiment, called passage 1 (P1), actually corresponded to passage 4.

At passage 26 (P26), a thermal stress was applied to all subcultures, in order to evaluate the influence of an environmental pressure on the evolutionary behaviour of the virus. For this purpose, five subcultures were exposed to 15°C (subcultures 1 to 5) for 24 h, whereas the five others were exposed to 28°C (subcultures 6 to 10) for 24 h. Cultures were then kept back to regular conditions until the experiment's end, i.e. passage 50 (P50).

Extraction of viral DNA

Genomic DNA from infected cell supernatants was extracted using MagAttract HMW DNA Kit (QIAGEN® products), following the manufacturer's instructions. Before beginning the procedure, frozen samples were thawed quickly at room temperature (25°C), and 200 µL transferred into 2-mL tubes. DNA was eluted with 100 µL ultrapure distilled water provided in the kit. DNA extracts from two replicates of each subculture were pooled together and concentrated using a vacuum concentrator to reach a total volume of 15 µL. The purity of the

obtained DNA was verified using a Nanodrop spectrophotometer (NanoDrop™ 2000, ThermoFisher Scientific). DNA was quantified by fluorometry (Qubit, ThermoFisher Scientific), and its quality was evaluated by agarose gel electrophoresis.

Detection of ORF150-del by qPCR

The presence of the ORF150-del genotype was verified by qPCR after each passage, following the protocol used in Klafack et al. (2019). The qPCR assays were run in triplicate with a LightCycler® 480 (Roche). Reactions mixtures contained 0.2 µL of 20 mM of each primer, 2.5 µL of 2X SYBR SensiFast master mix, 1.5 µL of H₂O and 0.6 µL of genomic DNA in a final volume of 5 µL. Two primer sets were used: one set targeting the ORF150 of CyHV-3 (TGAGGATGCAGAAGCAGTGGATGT and GGTAAGGGTAAAGCAGACCATC) and the other located inside the deletion (GCTGGACCTGTCACAATTCTAT and TGGTCCAGTAGACGGTTGA). Cycling conditions were identical for both primer pairs and consisted of an initial denaturation at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 10 sec with a single fluorescence measurement. A melting curve was applied to verify the specificity of the amplified products. It consisted of a denaturation at 95 °C for 5 sec, a renaturation at 65 °C for 60 sec and a heating step from 65–97 °C with a ramp of 0.1 °C per second and a continuous fluorescence acquisition. As tested previously (Klafack et al., 2019), the deletion can be confidently detected under two conditions: the ratio between Cq ORF150 and Cq deletion is significantly lower than 1, and the melting profile of both amplicons is correct.

Genomic library preparation and Oxford Nanopore sequencing

Full genome sequencing was realised for all subcultures at P15, P30 and P50. Genomic DNA libraries were prepared using the Rapid Barcoding Sequencing kit (SQK-RBK004, Oxford Nanopore Technologies). Briefly, 100-200 ng of purified DNA of each sample were adjusted with nuclease-free water to reach a total volume of 7.5 µL. Each DNA was tagged with the addition of 2.5 µL of fragmentation mix (RB01-RB10) followed by two successive incubations of 1 minute at 30°C and at 80°C, and immediately placed on ice. The tagged DNA libraries were then pooled by five, and an equal volume of AMPure XP Beads (Beckman Coulter) was added. After a 5-minute incubation at room temperature, DNA was bound to

magnetic beads, washed twice with 70% (v/v) fresh ethanol, and eluted with 10 μ L of 10 mM tris-HCl buffer pH 7.5 supplemented with 50 mM NaCl. The quality of the retained DNA fragments was evaluated using the Qubit fluorometer. One μ L of RAP buffer was added to each pool of DNA libraries. Finally, a mixture containing 34 μ L of sequencing buffer, 25.5 μ L of Loading Beads, 4.5 μ L of ultrapure water and 11 μ L of tagged samples was prepared and loaded onto R9.4.1 flowcells. A total of 6 flow cells were sequenced for 24 hours, each containing five barcoded samples. Sequencing runs were controlled with MinKNOW software version 0.49.3.7.

DNA sequence analysis and variant detection

For each sample, bases from raw FAST5 files with a pass sequencing tag were recalled using the high-accuracy model of ONT Guppy basecalling software version 4.0.15 which improves the basecalling accuracy. The obtained fastq files were filtered to keep reads with a length \geq 1 kb, in order to span large structural variants. After the mapping step using minimap2 (Li, 2018), the sequencing depth was calculated for each sample using the plotCoverage tool implemented in deepTools2.0 tool suite (Ramírez et al., 2016). Sequencing coverage was assessed with the bamCoverage tool from the same tool suite and normalized using the RPGC (reads per genome coverage) method.

To detect structural variants (SVs) in the viral genomes of all subcultures, a mapping step followed by BAM filtering was performed. Raw reads were mapped against the KHV-U (Genbank #DQ657948.1) reference genome with minimap2 (Li et al., 2018). The obtained BAM files were then filtered using the option '-F' of Samtools view (Li et al., 2018) with the flag "4" to keep only mapped reads and with the flag "0x800" to remove chimeric reads (inconsistent/supplemental mapping). The resulting filtered BAM files were used as input data for the SV caller, Sniffles v2.0.7 (Sedlazeck et al., 2018). Only SVs \geq 10 bp and supported by at least ten reads were kept in the final VCF files. A single nucleotide polymorphism calling was also performed on the same BAM files, using the snp command of Medaka v1.7.2.

KHV-U served as a reference genome because the isolate used in this study was suspected to belong to the European lineage (Pretto et al., 2013). However, since its degree of similarity was unknown, a guided de novo assembly of P1 genome was realised with the stitch

command of Medaka v1.7.2, and with KHV-U as reference. Variant and SNP calling was performed against KHV-U with the same tools as described above.

Statistical analyses

A one-way ANOVA was performed using GraphPad Prism 9.3.0 to assess the significance of differences in the number of variations observed between the two sets of subcultures (1-5 and 6-10). When appropriate, a Tukey post-hoc test was applied to identify differences between the sets of subcultures. A two-way ANOVA was also performed to assess the significance of differences in the total number of variations ($AF > 0.5$) between each passage. If significant, a Šidák test was applied.

Results

***In vitro* cell cultures and cytopathic effects**

CyHV-3 induced typical cytopathic effects (CPE) on CCB cells, i.e. cytoplasmic vacuolisation, cell rounding, cell detachment, and cell death. Infected cells displayed varying infection plaques, from small foci infection zones to large ones. CPE typically started at 2 to 3 days post-infection (dpi) with a few small plaques (Figure 2). At four dpi, plaques were larger and more frequent. After 5 to 6 days, cells developed syncytium and, after seven days, started to die and to detach from the flask.

After P38, CPE was different and appeared much earlier. Infected cells exhibited CPE after one day of infection, showing numerous small and large plaques covering all cell surfaces. Many large plaques appeared at two dpi, and hyper-syncytium started at 4 dpi. Many dead cells floated in the medium as early as five dpi. For this reason, infected cells were harvested at 5 dpi instead of 7 dpi, until the end of the experiment.

Main features of sequencing data

After removing reads smaller than 1 kb, the total number of reads for P1, P15, P30 and P50 averaged 66595, 41528, 87988 and 188379, respectively. The average length of sequences was 5335 kb for P1, 10661 kb for P15, 11184 kb for P30 and 10744 kb for P50 (Table 1). The proportion of mapped reads was comparable for P1 (99.72%), P15 (98%) and P30 (97%), but was much lower for P50 (25%). Overall, genomes were entirely covered by the sequencing data

with average depths of 1750X for P15, 3502X for P30 and 1296X for P50 (Figure 3). Within each series, the number of reads was not evenly distributed between the barcodes, and this resulted in large differences in terms of sequencing depth. For illustration, sequencing depth varied from 348X to 3944X in P15 samples (Table 1). However, all sequencing depths were sufficient to obtain full genome coverage and allow reliable genome-scale comparisons.

Accumulation of genomic variations along serial passages and effect of thermal stress

Comparison of the *de novo* reconstructed P1 genome with that of the reference KHV-U strain revealed a very low number of differences: 20 SNPs and 7 SVs. For this reason, all comparisons were made against KHV-U, and as a consequence, all genome coordinates provided in the following sections refer to those of KHV-U (Genbank #DQ657948.1). This allows a direct localization of variations on the KHV-U annotated sequence.

The distribution of SVs was relatively homogeneous along the reference genome, with higher abundance peaks observed around 45k, 90k, 180k and 220k, as well as in the terminal repeats (Figure 4a). The majority of SVs consisted of insertions and deletions, with a small proportion of duplications. There was a great number of small SVs that were shared by several subcultures. The number of detected SVs amounted to 368 in P15 subcultures, then dropped to 196 in P30, and increased again in P50 (567). The same pattern was observed when considering only SVs with an allele frequency (AF) above 0.5, suggesting that this result was independent from artefacts potentially caused by low sequencing depth. This resulted in an average number of SVs (with AF > 0.5) of 8.4, 3.6 and 15.3, respectively.

In comparison, the average number of SNPs detected with an AF greater than 0.5 was 23.1 for P15, 161.4 for P30 and 147.7 for P50. As for SVs, SNPs were homogeneously distributed throughout the genome, with no clear mutation hotspot (Figure 4b). Most of the SNPs with AF>0.5 were conserved along serial passages, and shared between subcultures. A two-way ANOVA carried out between subcultures 1-5 and 6-10 indicated no significant difference in the number of SNPs between these two groups at P30 and P50 (Figure 5). In contrast, the number of SVs that were shared between P15 and P30, or between P15 and P50, was very low, as illustrated in Figure 6 for subcultures 5 and 8. Although the reduction in the number of SVs between P15 and P30 was not significant, their high number in P50 suggests that the thermal stress applied at P25 interrupted this dynamics of SV accumulation. However,

the absence of difference between the two series of subcultures suggests that both thermal stresses induced the same reductive effect on the accumulation of SVs.

It is important to note that the SV and SNP profiles of samples P15.1 and P30.1, as well as of samples P15.6 and P30.3, looked highly similar, suggesting that these samples might have been mistakenly mixed together during library preparation. For this reason, these four samples were excluded from the present analysis. This error might have caused an overestimation of the total number of variations in P15 subcultures. In any case, the accumulation of SVs and of SNPs along passages showed a clearly different trend.

Structural variations in open reading frames

Many SVs accumulated in open reading frames. Most of the time, they consisted of small insertions or deletions located within variable numbers of tandem repeats (VNTR). However, very large insertions, up to 16 kb, could also be observed. Sequences of these insertions were of varying nature: some had a high similarity with *Cyprinus carpio* DNA; others corresponded to duplications of other portions of CyHV-3 genomes. The most intriguing ones were those that did not return any similarity with any known sequence. Generally, they had a high GC content, and they did not encode proteins. In all cases, these large insertions were found with a frequency $> 0,5$ in one subculture only, and were absent in P50, suggesting their random and probably deleterious character (Table 2).

Focus on the ORF150 region

Analysis of a 4-kb window spanning the ORF150 revealed a total absence of SNP and SVs in this region, whatever the subculture. The ORF150 deletion was not found.

DISCUSSION

Mutation during *in vitro* replication is a way for virus evolution that can be associated with random genetic drift or intentional bottlenecks (López-Muñoz et al., 2021). Despite their proofreading and repair mechanisms, herpesviruses accumulate SNPs during host cell passages. Furthermore, inter-genomic recombinations and SVs acquired during *in vitro* replication also contribute to the evolution of herpesviruses and other large DNA viruses (Weller et al., 2012 ; Pérez-Losada et al., 2015 ; Cudini et al., 2019 ; Kuny et al., 2020). In the present study, SNPs

seemed to accumulate faster than SVs in the genome of CyHV-3, leading to a high rate of genetic diversity. SNPs showed a higher stability, as the vast majority had an AF above 0.5 and were shared between subcultures. Their accumulation rate was, however, higher than what had been observed previously with a CyHV-3 isolated in Taiwan (Klafack et al., 2019). Though the previous study relied on short-read sequencing, which is reputed as more reliable (Avarre, 2020), the presence of 10 replicate subcultures combined with the high read depth strengthened the present data. It is important to note that only SVs with a size above ten nucleotides were considered in the present study, thereby excluding all short indels, which might be numerous. The consensus definition of a structural variation establishes a minimal length of 30 or even 50 nucleotides (Emerson and Cook, 2020). If this certainly reduced the observed diversity of the genomes, it yet allowed to concentrate on the big events only. The number of recorded SVs was smaller than that of SNPs. Of the 368 SVs detected in P15 subcultures, 34% had an allele frequency above 0.5, i.e. were dominant in the viral population. Moreover, in contrast with SNPs, the number of SVs continued to rise until P50. These results suggest the ability of CyHV-3 to generate variability in cell cultures through SVs quickly and continuously.

As for other herpesviruses, this indicates an adaptation of CyHV-3 to cell culture associated with genome alterations in the absence of selective pressure (Hedrick et al., 2000 ; Boutier et al., ; Schroeder et al., 2019). The impact of genetic changes depends on factors such as the location of the variations (inside/outside an ORF) and the scale of the changes in the genome (Lee et al., 2015). Some beneficial mutations in CyHV-3 have been reported to improve survival or replication; the process of natural selection may cause the mutation to become more common over time.

Selection is imposed by the host's immune system, environmental factors, and antiviral treatment (Hwang et al., 2004). Substantial evidence showed the relationships between temperature and the transmission and propagation of many viruses, including CyHV-3 (Kantzow et al 2016 ; Xin et al 2020). Natural selection, such as that triggered by ambient temperature changes, is widely known to influence the evolution of viruses (Nifong and Gillooly, 2016 ; Murrieta et al., 2021). The extent of selective pressure exerted on a virus in its host environment can significantly shape the accrual of mutations (Szpara et al., 2014).

To test this hypothesis on CyHV-3, a modification of the incubation temperature during the first 24 hours of infection was applied at passage 26. This was the simplest way to introduce

environmental stress, or pressure, in our experimental setting. Temperatures of 15°C and 28°C were applied because they are the extremes for cell permissivity to CyHV-3 and also the extremes that CCB cells could tolerate without damage (reference and personal observations). The accumulation of SNPs was not affected by the thermal stresses applied at P26. In contrast, the lower number of SVs at P30 compared to P15, though not significant, tends to indicate that this type of variation was affected by the thermal stress. The absence of difference between the subcultures subjected to the two temperatures suggests that the introduction of a thermal stress in the culture conditions was sufficient to remove most structural variations, whatever its degree. Of note, the highest number of very large insertions occurred at P30. However, they were different from those recorded at P15, and were never found in more than one subculture. This may suggest that the thermal stress removed all of them, and that new ones appeared between P26 and P30. Interestingly, these large insertions totally disappeared at P50, probably pointing to a deleterious effect.

A striking result was the very low divergence of the Italian isolate used in this study with KHV-U: genome comparisons identified only 20 SNPs and 7 SVs. This isolate originated from Israël and was shown to belong to the European lineage (Pretto et al., 2013). However, it was sampled nearly ten years after KHV-U, which confirms that the evolution of CyHV-3 in its host is very slow (Hammoumi et al., 2016).

Sequencing results indicated that no deletion in the ORF150 region occurred at any passage. This finding contradicts what was expected from previous studies investigating variation accumulation along serial passages (Klafack et al., 2019 ; Fuandila et al., 2022). The present study focused on a CyHV-3 isolate that belongs to the European lineage, whereas the former used a Taiwanese isolate that belonged to the Asian lineage. If genetic differences have been highlighted between these two lineages (Aoki et al., 2007 ; Li et al., 2015 ; Hammoumi et al., 2016), yet little is known regarding potential differences in their genetic and phenotypic properties (Gao et al., 2018). Preliminary findings indicated that isolates belonging to the European lineage are less stable *in vitro* (Klafack et al., 2017), and may recover features of the Asian one after a couple of passages on cell culture, as was obviously the case for the GZ11 isolate (Dong et al., 2011 ; Bergmann et al., 2020). Verification of the presence of the ORF150 deletion in this isolate over additional passages should therefore help to clarify this point. The

initial composition of the virus suspension and the cell culture medium may also have had an influence on the accumulation of variations (Amtmann et al., 2020).

The acceleration of CPE observed after passage 38 was probably correlated with an acceleration of CyHV-3 replication. The rapid expansion of the observed syncytial plaques, known to trigger necrosis or apoptosis, may be an indicator of increased viral fitness (Goh et al., 2016). Syncytium formation is one of several mechanisms allowing direct cell-to-cell transmission of viral material without requiring assembly (Cifuentes-Muñoz, 2018). Cell-to-cell transmission in herpesviruses has been associated with altered dissemination patterns, affecting disease manifestation (Yang et al., 2014). Syncytial plaque formation is typical in CyHV-3 strains adapted to cell culture, indicating better fitness in cell culture. Infectivity of CyHV-3 in CCB cells decreases after reaching the maximum titer, which generally occurs 4-5 days post-inoculation with progressing cell lysis (Mletzko et al., 2017). This loss of infectivity could be due to proteolytic enzymes released from the cells that affect the proteins in the viral envelope, and perturbs virus entry into the cells and consequently viral replication (Amtmann et al., 2020).

Genomic variants with contradicting fitness levels *in vitro* and *in vivo* correlated to an SNP in the ORF131, resulting in either alanine (183A) or threonine (183T). The 183A variant was associated with syncytial plaque formation and revealed higher fitness *in vitro* but lower *in vivo*, and vice versa (Gao et al., 2023). Previous studies also revealed that the strains with the highest fitness in cell culture were those associated with the most extended cell passage history and the lowest virulence *in vivo* (Gao et al., 2018). Interestingly, this specific mutation (corresponding to position 226228 on the reference KHV-U genome) was detected in all P30 and P50 subcultures as a homozygous variant (100% of the corresponding reads harboured the mutation). In contrast, this mutation was absent in P1, only detected in P15.6 as a minor variant and in P15.1 as a major one. This seems to confirm the findings of Gao et al. (2023) on another CyHV-3 isolate and may explain this shift in CPE observed at late passages (after P38), or at least part of it. However, we do not know the role that thermal stress played in the fixation of this mutation.

The cell culture method has been widely acknowledged for its versatility in designing various vaccines and has become a valuable model for investigating viral pathogenicity. Nonetheless, viral adaptation to the cell culture seems to be a common feature in CyHV-3. This

adaptation, which is associated with unintentional mutations during *in vitro* replication, seems to entail a tradeoff between the *in vitro* and *in vivo* fitness of CyHV-3 (Gao et al., 2018 ; 2023). Therefore, viral attenuation, which is often a consequence of such mutations, is prone to reversion. The commercial live vaccine Cavoy, made in Israel by KoVax Ltd., was withdrawn from the US market due to the risk of reversion to a virulent form (Boutier et al., 2015). Vaccine design should be a concern that requires precise information underlying the *in vitro* attenuation. Better vaccine design and optimisation require detailed information about the vaccine preparation and the vaccine's components, including genes or proteins. As mentioned in Meeusen et al. (2007), the precise removal of genes from a viral genome ensures it cannot revert to a virulent form. Consequently, it is crucial to compile insights into the *in vitro* genetic evolution of CyHV-3, mainly in the regions of the genome responsible for virulence and replication. This knowledge is required for developing effective prevention strategies against CyHV-3 infections.

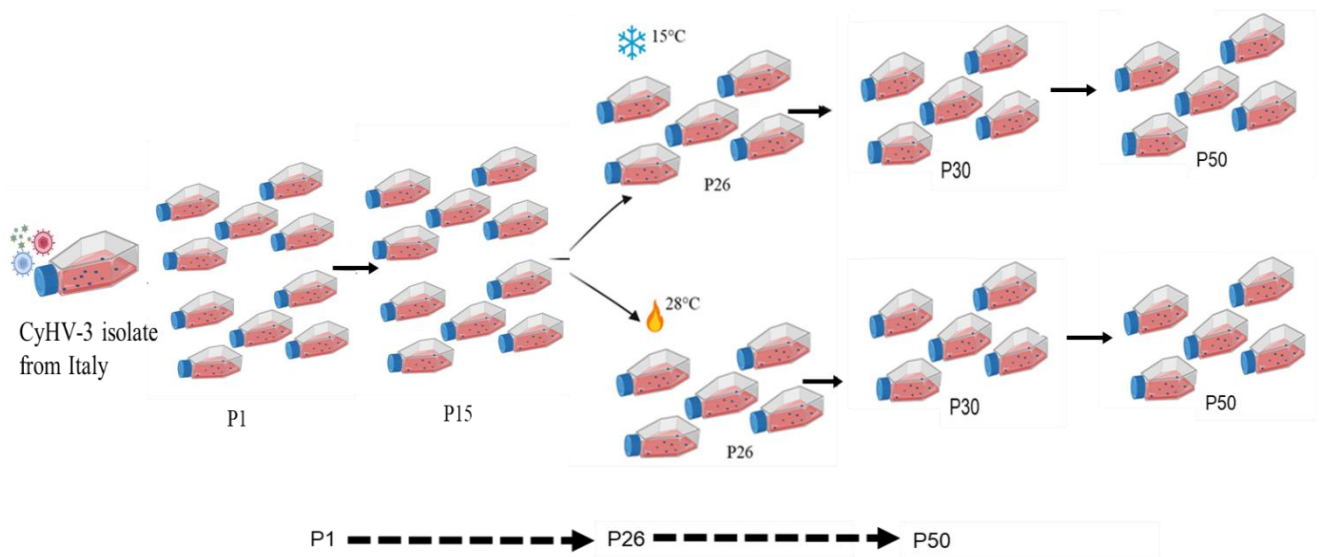


Figure 1: Experimental design. Ten flasks of 24-hour cells were inoculated with the same viral suspension, and serially passaged under the same conditions. A thermal stress was applied at passage 26 (P26): five subcultures were exposed to 15°C (1 to 5) for 24 h, whereas the five others were exposed to 28°C (6 to 10) for 24 h. Cultures were then kept back to regular conditions until passage 50 (P50).

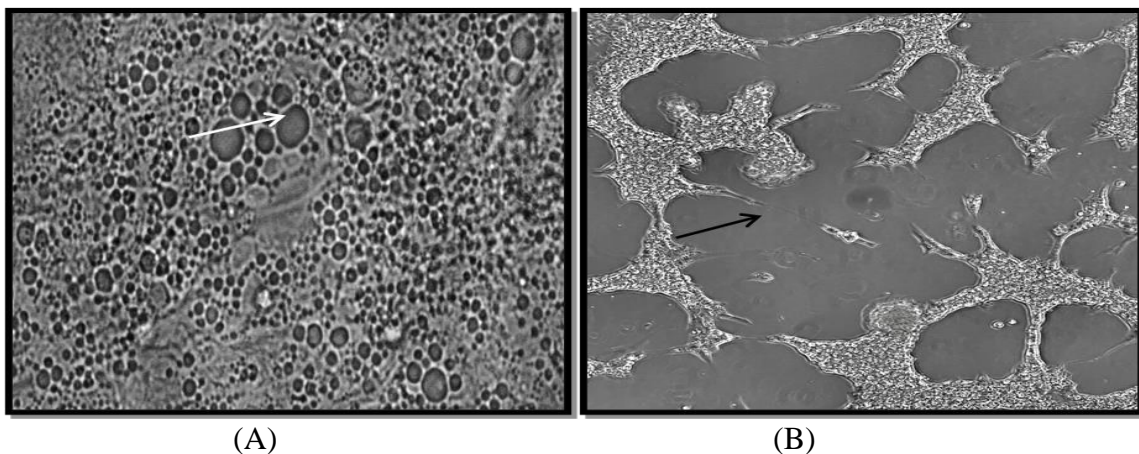


Figure 2: (A) CCB infected cell incubated at a permissive temperature of 24°C and CPE observed at P8 after 5 days p.i. The white arrow pointed to many small plaques and a few large plaques covering all cell surfaces. (B) CCB infected cell incubated at P38 and CPE observed after 5 days p.i. Most cells lost their adherence, as pointed out in the black arrow and showed abundant hypersyncytium (black arrow).

Table 1: Main features of sequencing data

Sample	Number of reads		Max length	Average length	N50	% mapped reads*	Average depths (X)
	Raw	> 1000		> 1000	> 1000		
P15-1	17338	14033	173755	8167.9	14245	98.99%	410.4
P15-2	12526	9833	150562	9989.7	17551	99.36%	348.3
P15-3	19073	15379	139181	12847	22845	98.82%	695.7
P15-4	55117	51854	160682	10942.5	16219	99.58%	2085
P15-5	17002	15829	160225	10719.1	15993	99.29%	619.6
P15-6	28189	22206	175683	11022.9	22163	98.34%	853.7
P15-7	76423	71778	149565	11680.1	17368	99.20%	3027.4
P15-8	58790	51001	133046	11731.9	19820	99.59%	2119.2
P15-9	81254	77083	134153	12223.4	18082	98.94%	3396.2
P15-10	90723	86293	161507	12610.9	18878	99.44%	3944.4
P30-1	38411	34116	193893	10123.7	16438	97.62%	1243.4
P30-2	126164	116513	214890	11401.6	17649	99.22%	4820.1
P30-3	41618	38678	190020	12414.3	20301	97.22%	1699.5
P30-4	106284	94083	148342	11436.3	20076	98.15%	3810.7
P30-5	123305	108902	145756	11568.6	19379	96.02%	4362.4
P30-6	138804	123218	164923	13371.1	22753	95.13%	5644.4
P30-7	114421	104804	116468	9201.8	13844	97.97%	3510
P30-8	90760	78674	106403	10392.6	17705	99.28%	2948.5
P30-9	96556	83072	179760	10951.7	19253	94.98%	3155.2
P30-10	111562	97826	139326	10978.6	18342	98.20%	3831.6
P50-1	140888	126040	137944	11318.2	18463	29.52%	1330.3
P50-2	202528	183886	172166	11477.5	18176	24.32%	1569.1
P50-3	210737	189634	137376	10143.7	16743	19.64%	1167.9
P50-4	477343	431977	123593	8739.9	13652	22.53%	2774.5
P50-5	50933	45242	129668	12810.4	21516	24.03%	425.8
P50-6	351907	319584	113195	9676.7	15231	18.42%	1892.7
P50-7	199772	184196	129118	11540.7	18261	19.00%	1240.8
P50-8	125181	109990	132825	10206.5	16647	14.31%	439.3
P50-9	231320	210043	132135	11310.4	18069	18.88%	1390.6
P50-10	93524	83199	116351	10219.7	16257	26.91%	733.8

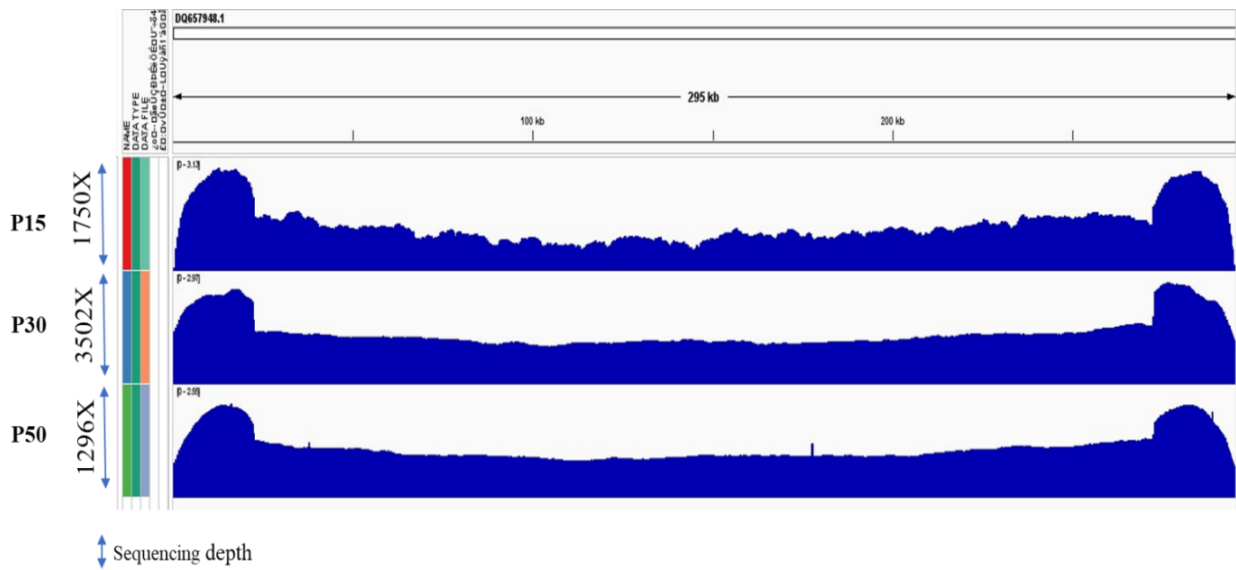


Figure 3: Visualisation of read depth and read coverage for 3 subcultures. The top panel displays the 295-kb KHV-U reference genome, and the bottom panel shows the mapping of the reads obtained for P15.1, P30.1 and P50.1. The provided sequencing depth is the average for the 10 subcultures of each series (P15, P30 and P50). Sequencing depth is higher at both genome extremities because these regions correspond to terminal repeats.

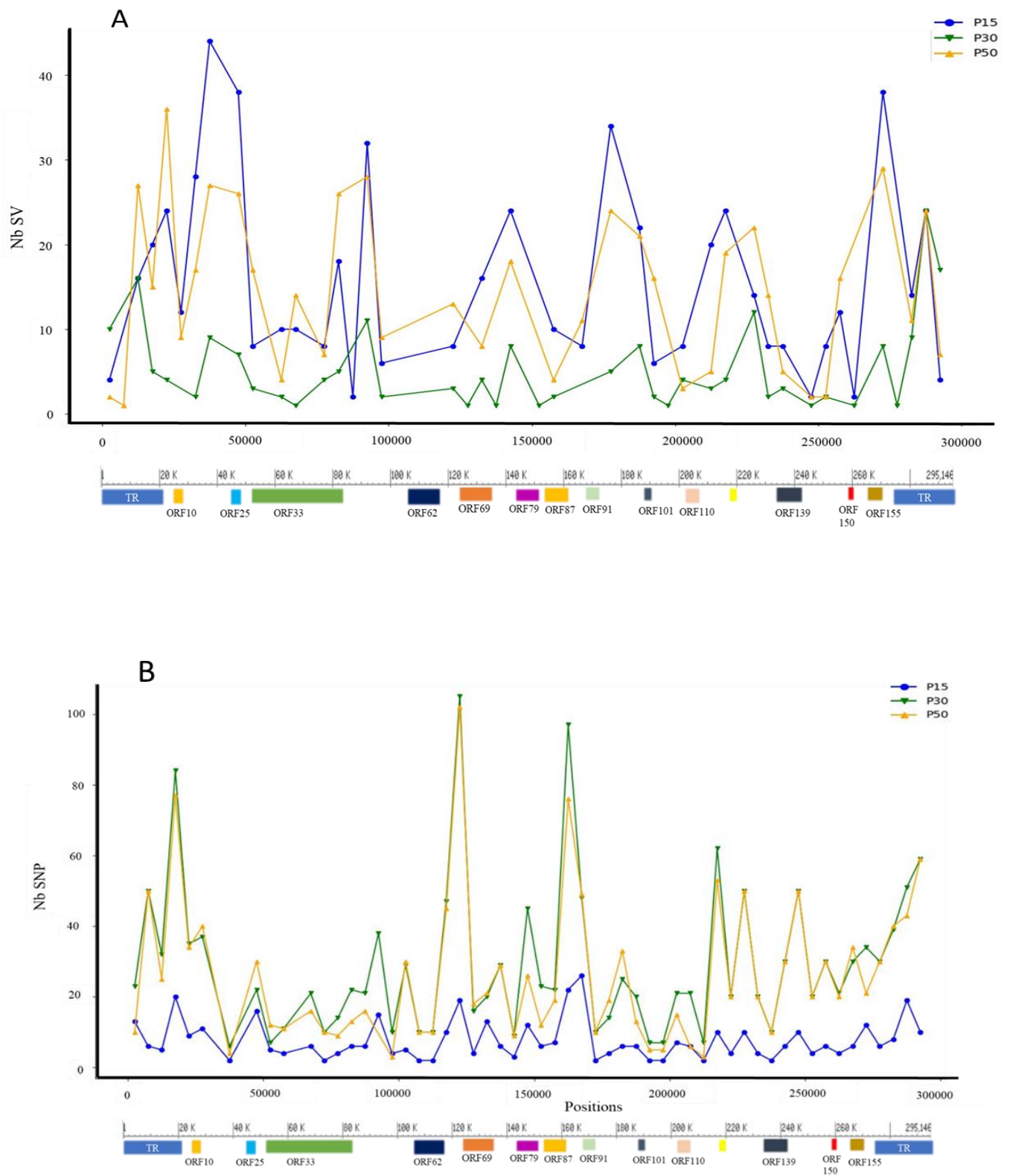


Figure 4: Number and position of SVs (A) and SNPs (B) against KHV-U reference genome.

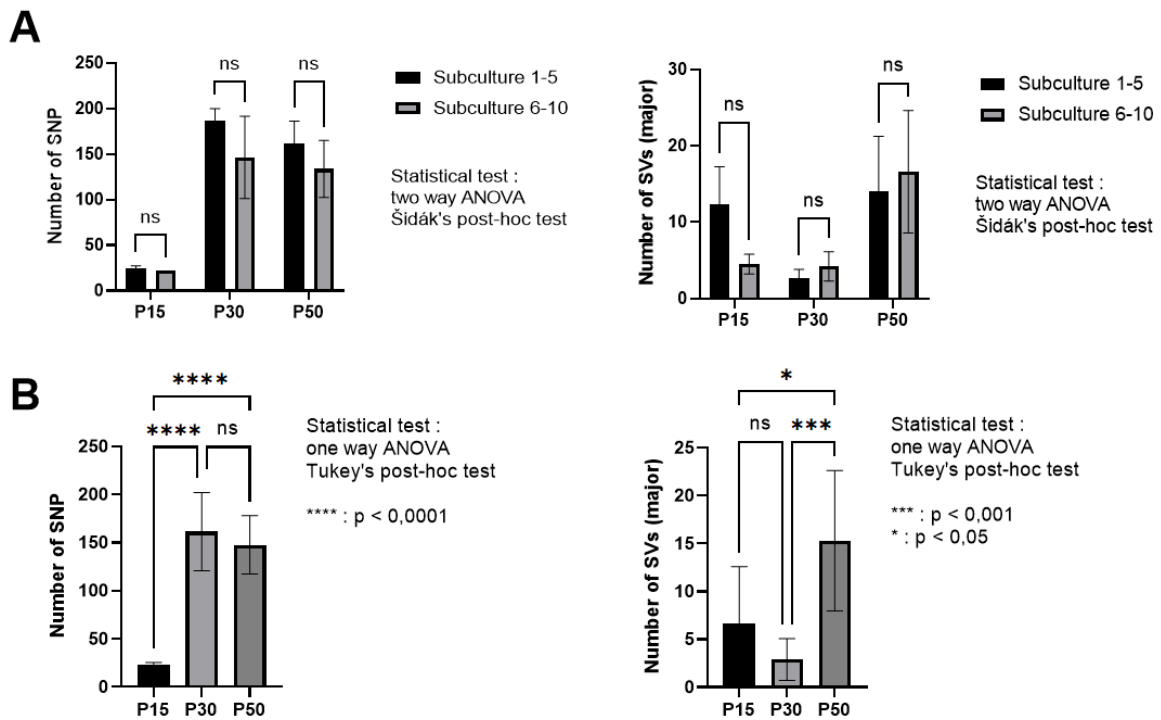


Figure 5: Changes in the average number of SNPs and SVs (A) between the three passages and (B) between the two series of subcultures (1-5 and 6-10). For each passage, the average number of SNPs or SVs was calculated (excluding passages P15.1, P15.6, P30.1 and P30.3). Only SNPs and SVs with an allele frequency greater than 0.5 were considered. Significance was tested by a Two way ANOVA or One way ANOVA (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non significant)

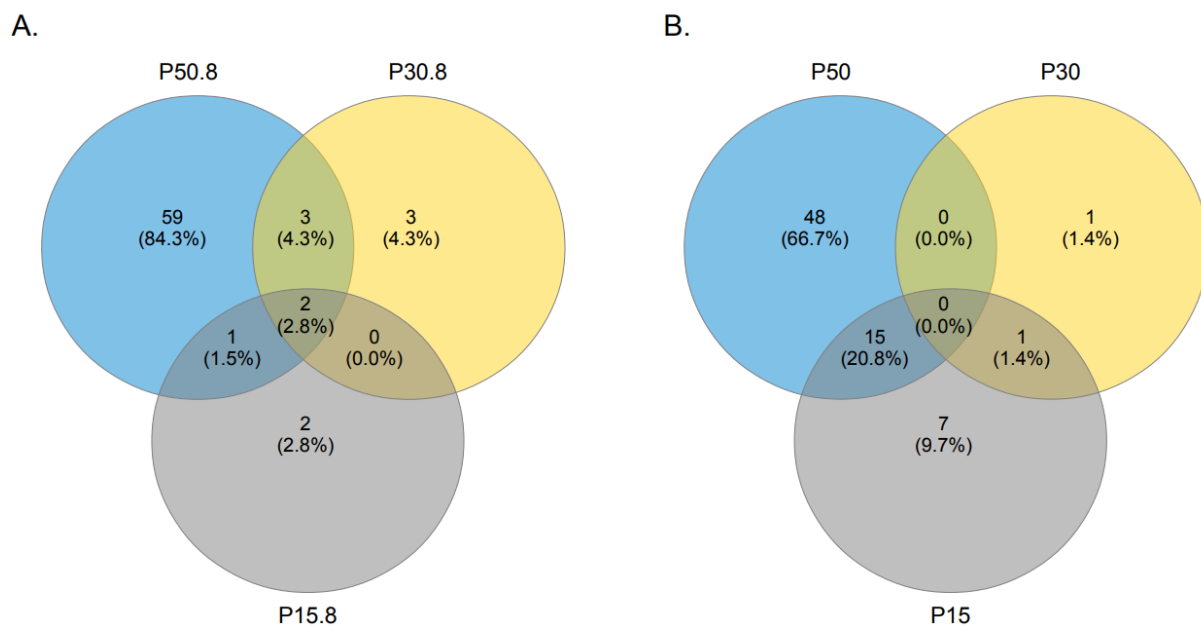


Figure 6: Conservation of SVs between passages for subcultures 8 (A) and 5 (B). Comparison was performed with *vcf compare v1.5* tool (Danecek et al., 2011), and Venn diagrams constructed with Rstudio (R Core Team, 2021).

Table 2: SVs present in putative ORFs, in at least one subculture and with an allele frequency greater than 0.5.

SVs present in at least 1 subculture and with an allele frequency greater than 0.5

SV present in this subculture SV absent in this subculture

	End	Length	Type of SV	Localisation	CDS	Comment	Subculture													
							1	2	3	4	5	6	7	8	9	10				
P15	11375	31	Insertion	ORF 6_1	No															
	31283	(-27)	Deletion		No		X	X		X										
	32540	29	Insertion	ORF 17	No		X	X	X	X			X	X						
	37879	76	Insertion	ORF 19	No	deoxynucleoside (deoxyguanosine) kinase	X		X	X			X	X	X	X				
	49343	(-75)	Deletion	ORF 27	No	ORF25 family; membrane glycoprotein	X	X												
	85419	7568	Insertion	ORF 49, 50, 51	Yes														X	
	95742	(-11)	Deletion		No		X	X		X										
	122251	5459	Insertion	ORF 65	Yes	ORF25 family; membrane glycoprotein													X	
	127207	(-12)	Deletion	ORF 68, 69	Yes	ORF68: Similar to myosin and related proteins and possibly to ICHV-1 ORF22	X			X										
	142608	6168	Insertion	ORF 75, 77, 78	Yes	ORF75: capsid maturational protease (Provisional)/ ORF78: Related to ICHV-1 ORF28; capsid protease and scaffolding protein														X
	143533	(-24)	Deletion	ORF 75, 77, 78	Yes		X	X		X										
	155512	-66	Deletion	ORF 84, 85	Yes			X												
	177614 - 177622	(-46) - (-52)	Deletion	ORF 97	No		X		X	X			X	X	X	X				
	178832	(-12)	Deletion	ORF 97	Yes		X	X		X										
	210837	(-27)	Deletion	ORF 116, 117	Yes	ORF116: Membrane glycoprotein	X													
	249095	10	Insertion	ORF 145	No		X	X		X										
	265177	12	Insertion		No		X	X		X										
	270660	(-15)	Deletion		No		X	X												
272771	3210	Insertion		No														X		
284052	31	Insertion	ORF 6_2	No		X	X		X									X		
P30	1210	12	Insertion	ORF 1_1	No					X				X						
	11417	(-33)	Deletion	ORF 6_1	No										X					
	14453	25	Insertion		No										X					
	37879	74	Insertion	ORF 19	No	deoxynucleoside (deoxyguanosine) kinase											X		X	
	54238	4977	Insertion	ORF 30, 31, 33	Yes	ORF30: Membrane glycoprotein/ ORF 31: Similar to eukaryotic DUF614 proteins/ ORF32: Similar to a family of Singapore grouper iridovirus proteins/PLAC8 Family						X								
	138818	8855	Insertion	ORF 70, 71, 72	Yes	ORF71: Related to ICHV-1 ORF25; DNA helicase ORF72: Capsid Triplex subunit 2 (Provisional)								X						
	155479	6668	Insertion	ORF 84, 85	Yes													X		
	177644	(-76)	Deletion	ORF 97	No										X					
	196282	16622	Insertion	ORF 105, 106, 107	Yes	ORF105: Among the least convincing protein-coding regions/ ORF107: Related to ICHV-1 ORF56	X													
	254597	4226	Insertion	ORF 148	Yes	ORF25 family; membrane glycoprotein												X		
	274024	6383	Insertion		No					X										
	277349	12039	Insertion	ORF 3_2	Yes									X						
	284094	(-33)	Deletion	ORF 6_2	No													X		
	287130	25	Insertion		No					X					X					
	295075	12	Insertion		No		X			X	X	X	X	X	X	X	X	X	X	

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6. General discussion and perspectives

The first analysis of CyHV-3 structural variations at the genome scale

Viral evolution depends on the availability of genetic diversity for fitness-based selection (Chen et al 2014). Each genome component (e.g. coding sequence, intergenic sequence...) experiences modifications across generations due to the interplay of various evolutionary mechanisms. If nucleotide substitutions are a major contributor to producing new variants, they are however not the only source of viral diversity. Genomic rearrangements, which result from recombination and can lead to the insertion of heterologous genetic sequences, gene duplications and deletions, are also an important player (Leung et al., 2017).

Compared to the family *Orthoherpesviridae*, there are relatively few studies regarding evolution within the family *Alloherpesviridae* (Waltzek et al., 2005, 2009 ; Aoki et al., 2007 ; Davison et al., 2013 ; Gao et al., 2018 ; Klafack et al., 2019 ; Gao et al., 2023). Recent results of Donohoe et al., (2021) revealed less genetic diversity within *Cyprinivirus* species clades than within species in the family *Herpesviridae*, which may be linked, at least in part, to different environmental pressures. Results described in the first chapter show that genomic rearrangements massively occur during CyHV-3 *in vitro* replication, possibly representing an underestimated source of genomic diversity. Though the mechanisms of SV formation are not well known, they likely involve recombination (Szpara and van Doorslaer, 2021 ; Tomer et al., 2019), which is often linked to replication and DNA repair, as well as errors during viral genome replication (Kulkarni and Fortunato, 2011 ; Xiaofei and Kowalik, 2014). This was the first time that structural variations were analysed in this virus at the genome scale. SVs mainly consisted of deletions and inversions, and their number was surprisingly high. Their allele frequency was often low, but could rapidly increase or decrease over cell passages. The occurrence of multiple viral haplotypes is an evolutionary advantage since lethal or sub-lethal viral haplotypes could be maintained in the population, thanks to genetic complementation, and be an additional reservoir of continuous genetic variation (Segredo-Otero and Sanjuán, 2019). Variants in viruses confer adaptive benefits in response to new environmental conditions, host immune defenses and antiviral treatments (Kuny et al., 2020). Variation during *in vitro* replication is a way for the evolution associated with intentional bottlenecks, such as plaque purification or the generation of mutant subclones (Parsons et al., 2015 ; McCrone and Lauring,

2018 ; López-Muñoz et al., 2021). As previously reported, most clinical and laboratory-grown isolates of herpesvirus show extensive genome alterations (Szpara and van Doorslaer 2010 ; Diakidi-Kosta et al., 2003). Therefore, investigating the ways SVs accumulate in viral genomes can unveil insights into the evolutionary strategies of viruses to ensure their survival and persistence, shedding light on their complex biological cycles.

Sequencing can readily be adapted to study the population dynamics of CyHV-3 and evaluating the presence of new variants. As exemplified for CyHV-3, in chapter 1, advanced sequencing technologies provide new opportunities to study these mechanisms at the genome scale. This technology has been successfully used for monitoring outbreaks for diagnostics, or for studying virus transmission in other aquaculture-impactful viruses (Gallagher et al., 2020a) ; Bayliss et al., 2017 ; Macqueen et al., 2021 ; Morga et al., 2021). It should be kept in mind that the diversity of viral haplotypes identified for each passage, in the present study, is certainly not representative of the assembly that is finally transmitted to the next passage. In fact, we sequenced viral genomes isolated from cell cultures showing CPE, which include both viral genomes present in viral particles of the cell supernatant and those released by the lysed cells. Thus, of all the haplotypes identified, some were intracellular and not necessarily intended to be encapsidated. It would therefore be interesting to carry out the same type of experiment using an approach based on Virion-Associated Nucleic Acids (VANA) sequencing (Filloux et al., 2015).

Cell culture for investigating the role of viral ORF in CyHV-3 replication and virulence

One way to maintain the health of aquatic organisms is immunisation. This approach has been considered a sustainable one to disease control by increasing the adaptive host immune system. Several studies are increasingly facilitating the rational design of attenuated vaccines against CyHV-3 by targeting various open reading frames (ORFs) (Meeusen et al., 2007). Discovering immunogenic proteins of CyHV-3 that are relevant can contribute to the enhancement of vaccines and the creation of more effective diagnostic tests (Fuchs et al., 2014). Some studies have explored several ORFs within CyHV-3 to understand their implication in virulence and replication (Boutier et al., 2017 ; Fuchs et al., 2011). In spite of this, most of CyHV-3 ORFs lack functional annotations. This is the case of the ORF150. Therefore, more

research efforts should be deployed to uncover the function of CyHV-3 ORFs, and their interactions with host proteins. This is required to develop efficient prevention strategies of CyHV-3 infections. Cell culture is a relevant and valuable tool for investigating pathogenicity of viruses, and even the potential interactions with the host immune response (Dolskiy et al., 2020 ; Wu et al., 2022 ; Das et al., 2023). It has been widely acknowledged for its versatility in designing various vaccines, including inactivated, subunit, vector, DNA and recombinant attenuated vaccines (Costes et al., 2008). Attenuated CyHV-3 vaccines were developed by continually disseminating CyHV-3 isolates with reduced virulence or low concentrations of the virulent virus in cell culture (Boutier *et al.*, 2017 ; Schröder et al., 2019). In the present case, cell cultures combined with whole-genome sequencing proved efficient in obtaining an attenuated vaccine candidate and identifying the putative ORF responsible for this attenuation. Generation of different engineered ORF150 deleted mutants confirmed that the full deletion of ORF150 offered a safe and efficient vaccine via oral administration or immersion (Klafack et al., 2022). Though this work was concomitant with the present one, it shows the importance of studying the genotype-phenotype interactions, and the role cell cultures may play. However, as for most ORFs, the exact function of ORF150 is still unknown, and further research is needed to better characterise the potential long-term effects of such a vaccine. ORF150 was shown to be transcribed at two hpi and was classified into one of the early genes (E), following an infection at permissive temperature (22 °C) (Ilouze et al., 2012a). ORF150 encodes a protein with a Really Interesting New Gene (RING) domain (Li et al., 2015 ; Aoki et al., 2007). This RING domain contains a structure that would be involved in the ubiquitination pathway, by acting as an E3 ubiquitin-protein ligase (He and Kwang 2008). Viruses that encode RING finger like-ubiquitin ligases (E3s) may evade host immune responses and also hijack the host's RING E3 to enhance their replication (Zhang et al., 2018). In aquatic viruses, RING family genes have been reported to be involved in host-virus interaction, virus latency, replication, and host protein degradation (Heilingloh et al., 2014 ; Shekar and Venugopal, 2019 ; Wang et al., 2021). ORF150 would contribute to suppressing carp's immune system to facilitate viral replication (Klafack et al., 2022).

***In vivo* genomic variations in the ORF150 region**

In contrast with *in vitro* infections, sequencing of *in vivo* samples showed a limited rate of genetic diversity in the ORF150 region. These findings suggest that ORF150 is not prone to frequent structural variations in natural populations. These results contrast with what was observed *in vitro* on the CyHV-3-T isolate and suggest that the genomic plasticity of CyHV-3 is lower *in vivo* than *in vitro*. They indicate that CyHV-3 multiplication *in vivo* may be limited enough to facilitate its clearance through innate immune response. The host's natural immune response probably constrains the spread of CyHV-3 by limiting its viral replication. It also probably acts as a selective pressure to remove any SV that would be deleterious, or that would lead to a decreased fitness *in vivo*. From the results described in chapter 1, we hypothesised that this deletion could play a role in the modulation of CyHV-3 virulence *in vivo*. The results presented in chapter 2 tend to indicate that this is not the case. However, they were obtained on a limited number of samples, and they require to be confirmed on a larger set of animals. Moreover, it cannot be ruled out that ORF150-del was not detected because of technical limitations, making it challenging to determine whether ORF150 deletion was really absent or present in light proportions. We made the choice to take a small piece of gill instead of sacrificing the fish for taking the spleen or liver, that would have contained more viral particles. This points to the difficulty of obtaining enough viral DNA to generate reliable sequencing results (Nkili-Meyong et al., 2017 ; van Aerle and Santos, 2017). Of the many CyHV-3-positive samples, only five could be amplified with at least 1 of the 3 primer sets tested, leading to a 3.3-kb amplicon. Besides, it required two rounds of PCR to obtain enough DNA for sequencing. It is known that amplicon-based sequencing is prone to amplification biases towards the major variants (Kebschull and Zador, 2015 ; Illingworth et al., 2017). This was illustrated in Klafack et al. (2019), where the undeleted ORF150 variant could not be detected in P78, in spite of a high sequencing depth. Only the use of digital PCR, known to be less susceptible to sequence abundance, could detect the undeleted variant in P78. This latter was present at a rate of 0.054%, i.e. was nearly 5×10^4 less abundant than ORF150-del haplotype. In any case, the absence (or low level) of the deleted ORF150 suggests that this deletion may not be the sole factor influencing changes in CyHV-3 pathogenicity in its natural host.

The prevalence of CyHV-3 in Indonesia

The first outbreaks of CyHV-3 occurred in Indonesia in 2002. Fish Quarantine Agency Quality Control and Safety of Fisheries Products government has conducted inspections in all regions that show notifiable KHVD symptoms. Since the first outbreak, this disease has caused many losses in the carp industry in Indonesia. In some farms located in West Java, outbreaks can now occur 1 to 3 times a year in the same farming. Furthermore, such outbreaks are no longer limited to the rainy season, generally associated with temperature fluctuations. It was indeed shown, at least in the Jatiluhur and Cirata reservoirs, West Java, that high mortalities could also occur at a temperature up to 31 °C (Avarre et al., 2012; chapter 2).

Indonesia exhibits three primary common carp farming methods: earthen pond cultivation, floating net-cage farming, and concrete rearing ponds. Most farms are predominantly composed of household-managed ponds of small to medium scale. Recently, commercial enterprises that produced huge quantities of carp switched to tilapia commodities because KHVD caused devastating losses. These phenomena were found in East Java and South Sumatra, where many common carp ponds or floating net cages were converted to tilapia-rearing ponds.

The results presented in chapter 2 indicate that the prevalence of CyHV-3 in Indonesia is still high in many provinces. This was also reported by Amin et al. (2018). West Java farms experience frequent outbreaks and are dominated by intensive systems. The survey realised in 2022 showed that most CyHV-3-positive fish originating from semi-intensive systems looked healthy. This contrasted with the intensive farms, where high clinical signs were reported. In intensive aquaculture, changes in water quality can occur more quickly due to high feeding, resulting in the accumulation of organic matter in the form of feed residues and fish faeces. The increased density in the fish-rearing system and the lack of sanitary barriers create favourable conditions for the rapid transmission of pathogens, resulting in elevated mortality (Tolo et al., 2022). This points to a link between animal density and development of clinical symptoms, as has been proven for many other animal breeding practices (Krkosek et al., 2007). It therefore raises serious questions about the rearing model Indonesia wants to develop.

Although clinical symptoms were largely absent in most CyHV-3-positive cases, it was noted that many asymptomatic samples had previously experienced KHVD. This discovery aligns with the earlier examination that fish that survived a first exposition to the virus might

carry it as a latent state (Uchii et al., 2009). The virus can be reactivated when environmental conditions change (Cano et al., 2020).

Effect of temperature on virus replication and pathogenicity

A significant difference in the outcome of all herpesvirus infections in poikilothermic hosts is related to their temperature dependency both *in vitro* and *in vivo* (Van Beurden et al., 2012). Among other water parameters, temperature is a principal environmental factor in virus replication and pathogenicity (Rakus et al., 2017 ; Cano et al., 2020). During the sampling campaign realised in 2022, frequent temperatures in common carp farming exceeded the permissive temperature range, from 28 °C to 31 °C. We speculated that the virus is well-adapted to the high temperatures of Indonesian waters. Nonetheless, these conditions were related to the persistence of many asymptomatic fish. Suboptimal water temperatures recreated a role in prolonging the latent phase of CyHV-3 infection, as observed in previous studies (Eide et al., 2011 ; Kim et al., 2020). However, other reports showed that CyHV-3 reactivation may not occur in the presence of permissive temperatures alone (Fujioka, 2015 ; Uchii et al., 2014), where outbreaks have seasonal dynamics (Tolo et al., 2022).

A variant conferring enhanced fitness *in vitro*

CyHV-3 strains adapted to cell culture tend to form syncytial plaques in laboratory conditions, indicating better cell culture growth. The presence of variants with contradicting fitness levels *in vitro* and *in vivo* was correlated to an SNP in the ORF131 coding sequence. This T>C substitution at position 226228 (according to KHV-U reference sequence, #DQ657948.1) results in the replacement of a threonine (183T) by an alanine (183A) residue. The 183A variant was associated with syncytial plaque formation and revealed higher fitness *in vitro* but lower *in vivo*, and *vice versa* (Gao et al., 2023). In this study, authors applied a kind of purifying selection by initiating interference at the cellular level *in vitro* or by inducing an elevated host resistance to superinfection *in vivo* (Gao et al., 2023). Through the natural selection process, the least fit variant was quickly eliminated during lytic coinfections, both in experimental conditions and within living organisms. The same mutation could be observed in the experiment described in chapter 3. Though absent in P1, it started to be detected in two P15 subcultures and was present in all subsequent subcultures from P30 onwards. The independent fixation of this mutation in the CyHV-3 genomes of all subcultures after P30 may be associated

with the sudden change in pathogenicity observed at P38, which resulted in quicker and more acute CPE. However, it might not be the sole factor that contributed to this change, for three reasons: i) the CPE switch occurred many passages later; ii) a quick analysis of CyHV-3-T sequencing data revealed that this mutation (a C instead of a T) was present in both P1, P78 and P99, which yet induced significant different *in vivo* outcomes (Klafack et al., 2019); iii) the mutation was also present in the reference KHV-I genome (Aoki et al., 2007). It is therefore tempting to speculate that some structural variants may have also contributed to this phenotypic change, especially among those that are shared by all P50 subcultures. To verify this, we need to follow their dynamics in all intermediate passages between P30 and P40, and make correlations with CPE. The format of this experiment, using 10 replicate subcultures, is well suited to identify such potential additional contributors. The results presented in chapter 3 also indicate that, contrary to SNPs, SVs continue to accumulate after passages. A large majority of these SVs were located in tandem repeats. These can be produced as a result of polymerase stuttering, which leads to the same motif being replicated several times. The other structural variations may be due to recombination mechanisms, particularly favoured by the rolling circle replication mode of herpesviruses (Pérez-Losada et al., 2015).

The absence of ORF150-del in the Italian CyHV-3 isolate, over 10 replicates, and even after a thermal stress, suggests that this deletion arised randomly in CyHV-3-T. However, the high instability of the ORF150 region observed in CyHV-3-T may also be a specific feature of the Asian lineage. This could be verified by doing the same *in vitro* experiments with another Asian isolate. There is indeed growing evidence that these two lineages share different properties, both *in vitro* and *in vivo* (Dong et al., 2013 ; Li et al., 2015 ; Klafack et al., 2017. Finally, the absence of ORF150-del in carp and in the Italian CyHV-3 isolate indicates that this deletion alone is not sufficient to regulate the virulence of CyHV-3. The absence of ORF150-deleted haplotype in both *in vitro* serial passages of CyHV-3-I and carp populations also suggests that this genotype might be used as a promising vaccine strategy to eradicate the disease and is suitable for mass vaccination of fish at the farm level. Additionally, vaccinated and non-vaccinated fish could be distinguished to monitor in more detail the efficacy of this vaccine in mass culture production (Klafack et al., 2022).

My thesis work contributes to the understanding of the evolutionary mechanisms of Alloherpesviruses, and of CyHV-3 in particular. The results suggest that SVs play a substantial

role in the evolution of CyHV-3 at least *in vitro*, when environmental pressures are absent. This kind of genomic studies provide the information to design preventive strategies and control the spread of pathogens, especially in vaccination development. They highlight the value of basic virology research in answering applied questions. Finally, collaborative efforts involving multiple stakeholders and support from the Quarantine Agency Quality Control will be required to promote sustainable aquaculture in Indonesia.

7. Résumé détaillé de la thèse

Introduction

Les poissons représentent la première source de protéines pour de nombreuses populations, puisqu'ils fournissent à 3 milliards de personnes près de 20 % de leur apport quotidien en protéines animales. L'aquaculture est le secteur d'élevage qui a connu la croissance la plus rapide au cours des dernières années. Elle procure aujourd'hui 50 % des poissons consommés dans le monde et joue donc un rôle de premier plan pour le maintien des stocks de poissons sauvages, pour la sécurité alimentaire et pour la subsistance de millions de personnes. Cette intensification a entraîné en contrepartie une multiplication des épidémies à l'échelle mondiale.

La carpe commune, *Cyprinus carpio*, est l'un des principaux poissons cultivés, non seulement pour la consommation humaine, mais aussi à des fins ornementales, la carpe koi étant très recherchée par les collectionneurs. Cette espèce fait partie de la famille des cyprinidés, la plus importante famille de poissons d'eau douce. Les maladies virales constituent une des principales menaces pour l'élevage de la carpe commune. L'herpesvirose de carpe (Koi Herpesvirus Disease, KHVD) figure au premier rang de celles-ci. Son agent étiologique, le *Cyprinid herpesvirus 3* (CyHV-3), récemment renommé *Cyivirus cyprinidallo3*, a été décrit pour la première fois en 1996 dans plusieurs pays simultanément. Il s'agit d'un grand virus à ADN double brin de 295 kbp, et l'un des virus aquatiques les plus pathogènes. Il infecte principalement la carpe commune et la carpe koi, et provoque des mortalités massives pouvant atteindre 100 %.

L'évolution des virus résulte d'un compromis entre diversification génétique et sélection naturelle. Bien que les mutations ponctuelles (SNP) ont longtemps été considérées comme les

principaux moteurs de diversité génétique, il est désormais admis que les variations structurales (VS) peuvent jouer un rôle important dans la diversité des virus. Les VS sont définies comme des réarrangements génomiques qui consistent en des délétions, insertions, inversions ou duplications, généralement de taille supérieure à 30 nucléotides. Le CyHV-3 existe dans son hôte sous la forme d'une population de variants génétiquement proches, ou haplotypes. Une étude antérieure réalisée sur un isolat de CyHV-3 provenant de Taïwan (CyHV-3-T) a montré que la composition de ces assemblages d'haplotypes peut rapidement varier au cours de passages en série réalisés en culture cellulaire *in vitro*, et entraîner des changements phénotypiques rapides. A l'issue de 100 passages consécutifs sur cellules CCB (common carp brain), des infections expérimentales avec les populations virales du passage 78 (P78) et du passage 99 (P99) ont révélé des niveaux de virulence différents par rapport à l'isolat d'origine : P78 était significativement moins virulent, tandis que P99 avait un profil de virulence intermédiaire. Le séquençage en lecture courte de ces différents passages a révélé une délétion de 1 363 pb dans l'ORF (Open Reading Frame) 150 de la majorité des haplotypes après 78 passages, mais une absence de cette délétion au passage 99. Ces résultats ont mis en évidence un lien phénotype-génotype, où l'ORF150 joue un rôle dans la virulence du CyHV-3 (Figure 1). La connaissance des mécanismes et des conséquences évolutives de cette diversité intra-hôte est essentielle pour développer des stratégies préventives, telles que des vaccins.

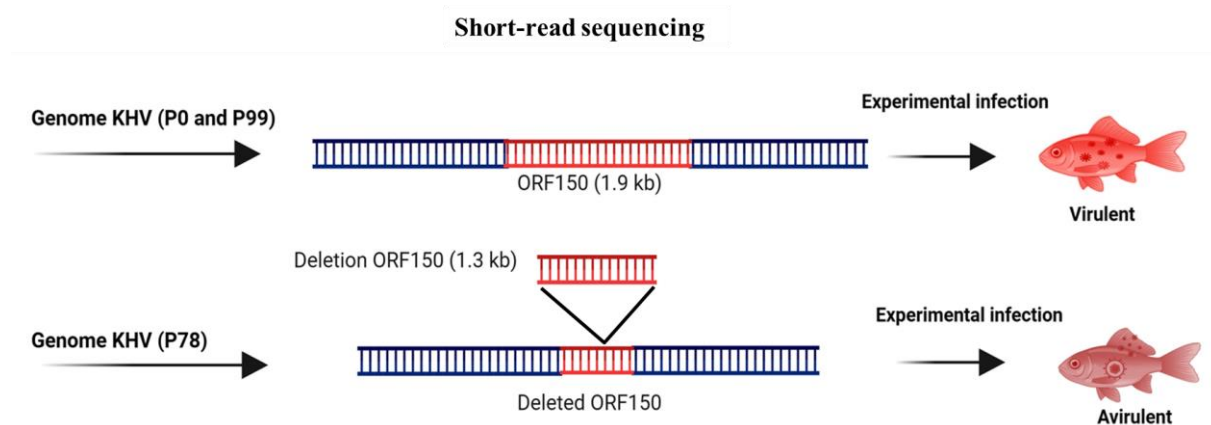


Figure 1 : Association génotype-phénotype mise en évidence par séquençage de passages viraux successifs sur cultures cellulaires. L'analyse comparative des différents passages a révélé

une délétion de 1 363 pb dans l'ORF150 après 78 passages, mais une absence de cette délétion après 99 passages.

Objectifs de la thèse

Ce contexte est à la base du présent travail de thèse, qui s'articule autour de trois objectifs principaux :

- 1) mieux comprendre les mécanismes évolutifs qui sous-tendent ces assemblages d'haplotypes en culture cellulaire, avec un intérêt particulier pour l'ORF150 ;
- 2) étudier la prévalence de la délétion ORF150-del et d'autres variations structurales dans cette région génomique *in vivo* ;
- 3) explorer les dynamiques d'accumulation des VS et des SNP dans le génome du CyHV-3 dans des répliques de passages en série sur cultures cellulaires et après l'application d'un stress thermique.

Résultats

Chapitre 1

Les travaux décrits dans le premier chapitre visent à mieux comprendre les mécanismes à l'origine de la présence et de l'absence de cette délétion de 1363 nt au cours des passages cellulaires. En utilisant le même isolat, CyHV-3-T, nous avons étendu l'analyse en étudiant les variations structurales au sein des mélanges d'haplotypes à P78 et à P99. Pour cela, du séquençage longs fragments a été utilisé pour la première fois sur CyHV-3. Afin de retracer la dynamique de gain/perte de l'ORF150-del au cours des passages successifs, nous avons également recherché les variations structurales au cours de dix passages intermédiaires (P10, P20, P30, P40, P50, P70, P80 et P90) en utilisant du séquençage sur amplicons. Au niveau de l'ORF150, le séquençage a confirmé que les ADN viraux du P99 ne contenaient aucune lecture contenant la délétion. Pour P78, la délétion a été trouvée dans presque 100% des lectures, tandis que l'haplotype de référence (non délété) a également été détecté dans 30 lectures, ce qui représente 0,44 % du nombre total de lectures.

Les résultats de séquençage ont révélé un nombre élevé de VS : 236 et 87 pour P78 et P99, respectivement. Pour comparaison, le nombre de petites variations (d'une taille < 30 nt) s'élevait à 57 et 77 pour P78 et P99, respectivement. Les inversions et les délétions étaient les formes

les plus fréquentes de SV, réparties sur l'ensemble du génome. Bien que la plupart de ces VS avaient des fréquences alléliques faibles, beaucoup étaient situées dans des ORF potentiellement impliquées dans la réplication et l'encapsidation de l'ADN, comme par exemple les ORF33, 46, 47 ou 55. L'analyse des passages intermédiaires a révélé la présence de l'haplotype ORF150-del à une faible fréquence dans les passages P10 à P40 (de 0,05 à 0,15%), puis une forte augmentation de sa fréquence à P50 (88,7%), pour atteindre un maximum à P78 (100%) et enfin chuter rapidement à P80 (30,7%) pour se stabiliser à une faible fréquence (0,31%) à P90 puis disparaître à P99 (Figure 2).

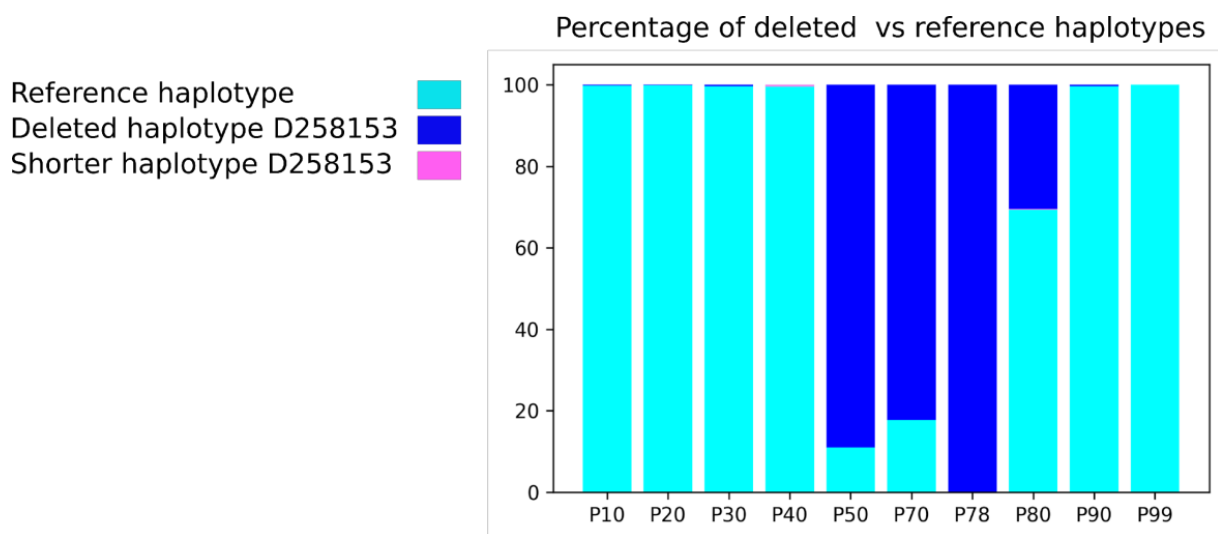


Figure 2 : prévalence de l'haplotype ORF150-del au cours des 10 passages intermédiaires (P10, P20, P30, P40, P50, P70, P78, P80, P90, P99) de CyHV-3-T sur cultures cellulaires CCB

Ces résultats ont montré que les VS étaient la principale source de polymorphisme de CyHV-3 dans ces cultures, et que ces dernières présentaient une dynamique de renouvellement élevée. Ces VS forment une mosaïque de sous-populations virales qui semblent résulter de multiples événements de réarrangement, principalement des inversions et des délétions. Certaines de ces variations peuvent conduire à la présence de génomes viraux défectifs dans cette mosaïque. Les résultats suggèrent que les changements rapides de VS et la présence de génomes défectifs représentent des facteurs clés dans la dynamique de la population virale et l'évolution *in vitro* du CyHV-3.

Chapitre 2

Le CyHV-3 peut donc évoluer rapidement au cours de ses cycles infectieux en système de culture cellulaire, qui se caractérise par l'absence de pression de sélection, tel que le système immunitaire. Les résultats décrits dans le premier chapitre amènent à se demander si l'accumulation de VS, et les changements rapides qui l'accompagnent, se produisent également *in vivo* et contribuent à la pathogénicité du virus. Pour répondre à cette question, nous avons étudié les génomes de CyHV-3 présents dans les élevages de carpes communes en Indonésie, dans des zones présentant différents niveaux d'endémicité. Compte tenu de la difficulté d'obtenir des génomes complets à partir d'échantillons *in vivo*, nous nous sommes concentrés sur la région de l'ORF150, très dynamique en termes de variations structurales. Nous avons combiné des analyses basées sur la qPCR et sur le séquençage long fragments pour détecter la présence du variant ORF150-del et d'autres variations structurales potentielles. Cette étude a également permis d'estimer la prévalence du CyHV-3 dans l'un des plus grands pays d'élevage de carpes qu'est l'Indonésie.

Une campagne d'échantillonnage de plusieurs semaines (Février-Mai 2022) a permis de collecter 236 poissons provenant de 43 élevages de carpes, répartis entre les îles de Java et de Sumatra. Cet échantillonnage non-invasif a consisté à prélever un petit morceau de branchies sur les poissons préalablement endormis. Les résultats de qPCR ont montré une forte prévalence du virus : 165 des 236 poissons échantillonnés étaient positifs pour le CyHV-3 (soit près de 70%) (Figure 3). De manière surprenante, seuls 66 poissons présentaient des signes cliniques sévères. En revanche, tous les poissons négatifs semblaient en bonne santé. La grande majorité des échantillons de Java Ouest étaient associés à des symptômes graves et la plupart présentaient un niveau d'amplification élevé.

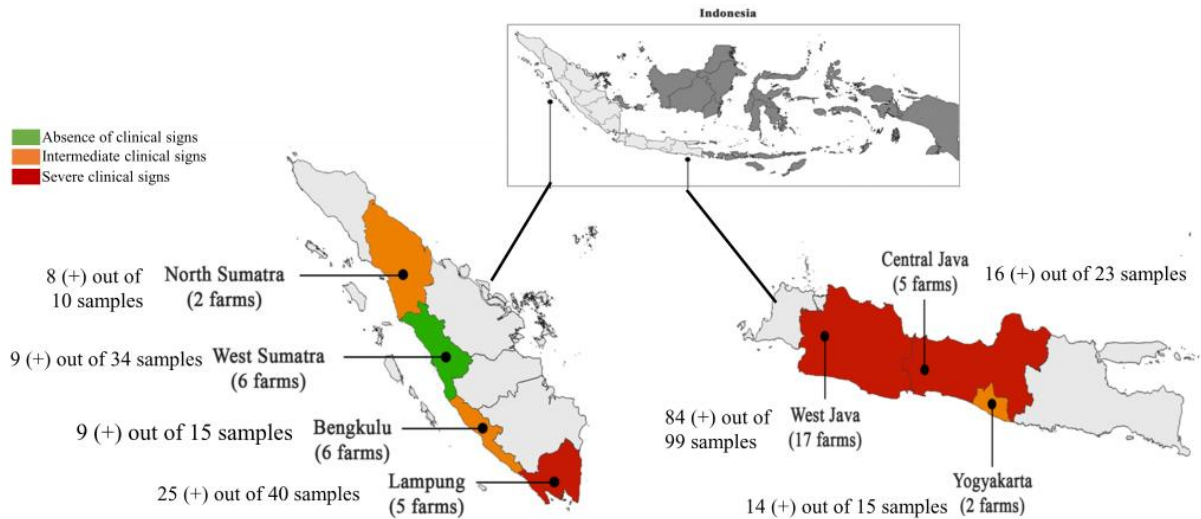


Figure 3 : Localisation des sites d'échantillonnage et prévalence du CyHV-3. Le code couleur indique la sévérité des signes cliniques observés.

La recherche de l'ORF150-del dans tous les échantillons positifs au CyHV-3, par qPCR, a montré que l'haplotype ORF150-del n'était présent dans aucun des échantillons d'Indonésie. Pour confirmer ce résultat, un séquençage longs fragments a été réalisé sur les échantillons qui présentaient les charges virales les plus élevées. L'analyse d'une région de 3,3 kb autour de l'ORF150 a révélé un nombre limité de variations structurales avec des fréquences alléliques très faibles (<1%). Elle a confirmé l'absence de la grande délétion de 1,3 kb dans l'ORF150. Ces résultats contrastent avec ce qui a été observé *in vitro* sur l'isolat CyHV-3-T et suggèrent que la plasticité génomique du CyHV-3 est plus faible *in vivo* qu'*in vitro*.

Chapitre 3

Les résultats obtenus dans le chapitre 1 ont montré que les SV peuvent jouer un rôle clé dans l'évolution *in vitro* du CyHV-3. Ceux obtenus dans le chapitre 2 ont indiqué, sur une région limitée du génome, et sur un nombre restreint de poissons, une plus faible diversité virale *in vivo*. Le chapitre 3 s'est donc intéressé à comprendre dans quelle mesure les assemblages d'haplotypes obéissent à un processus aléatoire (dérive génétique) et dans quelle mesure ils peuvent être influencés par une pression de sélection. Pour commencer à répondre à cette question, un isolat de CyHV-3, obtenu lors d'une épidémie en Italie (CyHV-3-I), a été propagé dans 10 sous-cultures de cellules CCB. Les populations virales de chaque sous-culture ont été passées indépendamment et en série 50 fois dans des conditions identiques. La température

ayant un effet important sur la virulence du CyHV-3, un stress thermique a été artificiellement appliqué au passage 26, au cours duquel cinq sous-cultures (1 à 5) ont été exposées à 15°C et les cinq autres (6 à 10) à 28°C. En utilisant le séquençage long fragments, nous avons évalué la nature et la fréquence des variations (SNP et les SV) qui se sont accumulées dans les génomes viraux entiers des sous-cultures aux passages 15 (P15), 30 (P30) et 50 (P50), c'est-à-dire avant et après le stress thermique.

L'analyse des séquences génomiques a révélé une distribution des VS relativement homogène le long du génome de référence. La majorité des SV se composait d'insertions et de délétions, avec une faible proportion de duplications. Le nombre moyen de VS (avec AF > 0,5) était respectivement de 8,4, 3,6 et 15,3 dans les sous-cultures P15, P30 et P50. En comparaison, le nombre moyen de SNP détectés (avec AF > 0,5) était respectivement de 23,1, 161,4 et 147,7 pour P15, P30 et P50. Comme pour les SV, les SNP étaient répartis de manière homogène sur l'ensemble du génome. La plupart des SNP avec AF>0,5 étaient conservés lors des passages en série et partagés entre les sous-cultures. En revanche, le nombre de VS partagées entre P15 et P30, ou entre P15 et P50, était très faible. Le stress thermique n'a apparemment pas eu d'effet sur la dynamique d'accumulation des SNP dans la population virale. En revanche, l'accumulation des VS était plus lente et manifestement affectée par le stress thermique (Figure 4).

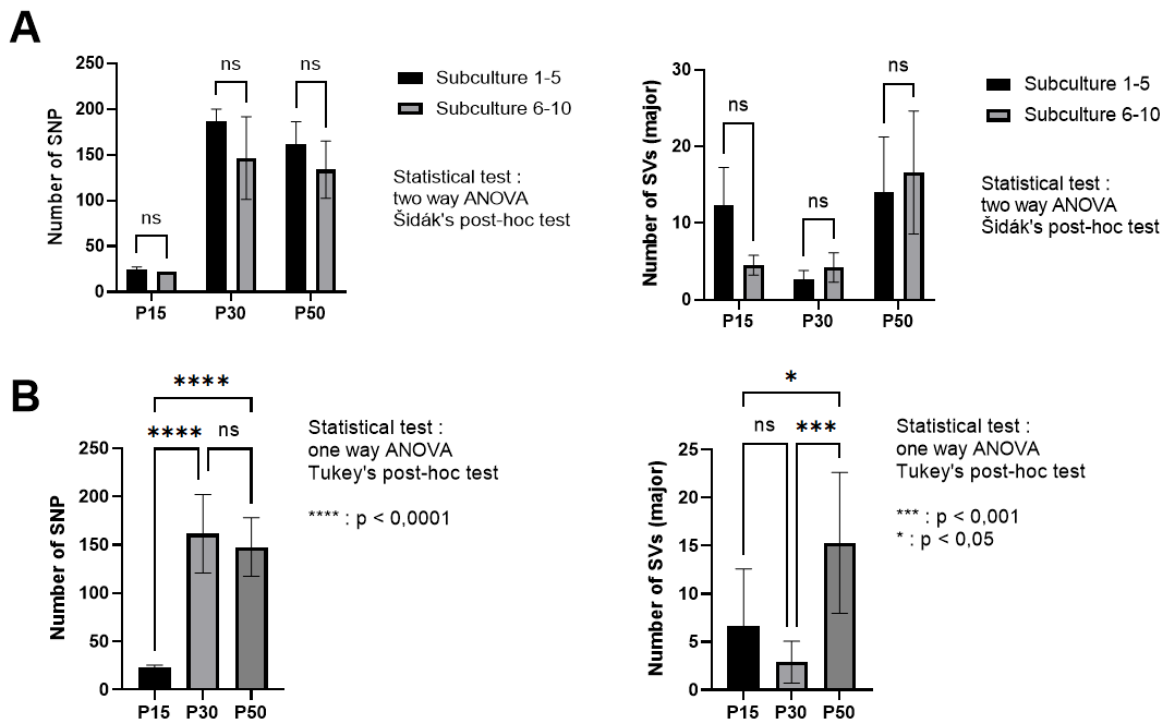


Figure 4 : Évolution du nombre moyen de SNP et de VS (A) entre les trois passages et (B) entre les deux séries de sous-cultures (1-5 et 6-10). Pour chaque passage, le nombre moyen de SNP ou VS a été calculé. Seuls les SNP et VS ayant une fréquence allélique supérieure à 0,5 ont été pris en compte. La significativité a été testée à l'aide d'une ANOVA (*, $p < 0,05$; ***, $p < 0,001$; ****, $p < 0,0001$; ns, non significatif).

L'analyse d'une fenêtre de 4 kb couvrant l'ORF150 a révélé une absence totale de SNP et de VS dans cette région, quelle que soit la sous-culture. L'haplotype ORF150-del n'a été détecté dans aucune sous-culture, même à très faible fréquence allélique. Aucune autre délétion dans l'ORF150 n'a pu être détectée, contrairement à ce que l'on pouvait attendre des résultats du chapitre 1. Enfin, les effets cytopathiques liés à l'infection se sont révélés plus sévères et plus précoces à partir du 38ème passage (P38). Cette observation a coïncidé avec l'apparition d'un SNP spécifique dans l'ORF131, dont une étude récente a montré qu'il était impliqué dans l'augmentation de la virulence du CyHV-3 *in vitro*.

Discussion générale et perspectives

Les résultats décrits dans le premier chapitre montrent que des réarrangements génomiques, ou variations structurales, se produisent massivement lors de la réplication *in vitro* du CyHV-3, et représentent probablement une source sous-estimée de diversité génomique. C'est la première fois que les variations structurales ont été analysées chez ce virus à l'échelle du génome. Bien que les mécanismes de formation des VS ne soient pas bien connus, ils impliquent probablement des mécanismes de recombinaison, qui sont souvent liés à la réplication et à la réparation de l'ADN. Il est communément admis que le mode de réplication du génome « haute fidélité » confère une relative stabilité génétique aux grands virus à ADN double brin, tels que les herpesvirus. Les travaux de cette thèse montrent que ce n'est pas le cas pour CyHV-3, pour lequel les SV semblent être un mécanisme évolutif important. Les variations confèrent aux virus des avantages adaptatifs en réponse à de nouvelles conditions environnementales, aux défenses immunitaires de l'hôte et aux traitements antiviraux. Par conséquent, l'étude de la façon dont les VS s'accumulent dans les génomes viraux peut permettre de mieux comprendre les stratégies évolutives des virus pour assurer leur survie et leur persistance, et mettre en lumière leurs cycles biologiques complexes. Comme l'illustre ce travail, les technologies de séquençage troisième génération (longs fragments) offrent de nouvelles possibilités pour étudier ces mécanismes à l'échelle du génome.

Les résultats présentés dans le chapitre 2 indiquent que la prévalence du CyHV-3 en Indonésie est encore élevée dans de nombreuses provinces. Les exploitations de l'ouest de Java connaissent des épidémies fréquentes et sont dominées par des systèmes intensifs. Les résultats indiquent un lien entre la densité des animaux et le développement de symptômes liés au CyHV-3, comme cela a été prouvé pour de nombreuses autres espèces d'élevage. La densité accrue dans les systèmes d'élevage de poissons, combinée à l'absence de barrières sanitaires, crée des conditions favorables à la transmission rapide de pathogènes, ce qui entraîne une mortalité élevée. La plupart des poissons positifs au CyHV-3 ne présentaient pas de signes cliniques ; d'après les producteurs, certains avaient déjà été victimes de la KHVD. Ceci est en accord avec le fait reconnu que les poissons ayant survécu à une première exposition au virus peuvent être porteurs de celui-ci à l'état latent. Le virus peut cependant être réactivé lorsque les conditions environnementales changent. Contrairement aux infections *in vitro*, le séquençage

d'échantillons *in vivo* a révélé un taux limité de diversité génétique dans la région de l'ORF150, et l'absence de l'haplotype ORF150-del. En limitant la propagation du CyHV-3, la réponse immunitaire naturelle de l'hôte agit probablement comme une pression sélective pour éliminer toute VS qui serait délétère ou qui conduirait à une diminution de la fitness du virus *in vivo*. Cependant, ces résultats nécessitent d'être confirmés sur un échantillonnage de poissons beaucoup plus grand.

La culture cellulaire est un outil pertinent et précieux pour étudier la pathogénicité des virus. Elle est largement reconnue pour sa polyvalence dans la conception de vaccins, au premier rang desquels les vaccins atténués. Ici, l'utilisation de cultures cellulaires combinée au séquençage du génome entier se sont révélées efficaces pour obtenir un candidat vaccin atténué, et identifier l'ORF responsable de cette atténuation. La génération de différents mutants au niveau de l'ORF150 a confirmé que la suppression complète de l'ORF150 offrait un vaccin sûr et efficace par administration orale ou par immersion. Bien que ce travail ait été réalisé en parallèle à cette thèse, il montre l'importance d'étudier les interactions génotype-phénotype et le rôle que peuvent jouer les cultures cellulaires. Comme pour la plupart des ORF du CyHV-3, la fonction exacte de l'ORF150 est encore inconnue, et des recherches supplémentaires sont nécessaires pour mieux caractériser les effets potentiels à long terme d'un tel vaccin. L'ORF150 code une protéine qui contient un domaine RING (Really Interesting New Gene). Ces protéines peuvent être impliquées dans l'interaction hôte-virus, la latence du virus, la réplication et la dégradation des protéines de l'hôte.

Enfin, l'absence de l'haplotype ORF150-del dans l'isolat italien CyHV-3-I du CyHV-3, sur 10 répétitions, et même après un stress thermique, suggère que cette délétion est apparue au hasard dans l'isolat taïwanais, CyHV-3-T. Toutefois, la grande instabilité de la région ORF150 observée dans le CyHV-3-T peut également être une caractéristique spécifique de la lignée asiatique. Cela pourrait être vérifié en réalisant les mêmes expériences *in vitro* avec un autre isolat asiatique. Il y a en effet de plus en plus de preuves que ces deux lignées présentent des propriétés différentes, à la fois *in vitro* et *in vivo*

Pour conclure, les résultats présentés ici suggèrent que les VS jouent un rôle majeur dans l'évolution du CyHV-3 au moins *in vitro*, en l'absence de pressions environnementales. Ils

indiquent que l'haplotype ORF150-del est apparu de manière aléatoire et mettent en exergue l'intérêt de la recherche fondamentale en virologie pour répondre à des questions appliquées.

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