Medical Microbiology

Thesis progression

Thesis title: Evaluating the emerging potential of zoonotic pox virus, ORFV by genomic characterization and molecular docking approaches

Thesis progression title: Confirmation of *Parapoxvirus orf* in suspected ORFV-infected samples using the PCR method

Student: Mr. Zwe Win Paing Student ID:677070008-3

Advisor: Assist. Prof. Dr. Supranee Phanthanawiboon

Date: 7th January 2026

---

1 Introduction

Poxviridae has two major subfamilies, including *Chordopoxvirinae* and *Entomopoxvirinae*. There are 18 genera in the first subfamily, and they are responsible for infecting vertebrates. The second includes 4 genera, which infect invertebrate organisms. The infectious species which have been infecting humans and causing significant death belong to the *Orthopoxvirus* genus. Variola virus (smallpox), monkeypox virus, and vaccinia virus are well-known for their large outbreaks in history. The other well-known viruses which can infect humans and have a high morbidity range within their primary hosts are Orf virus, Bovine papular stomatitis virus (BPSV), and Pseudocowpox virus (PCPV). The viruses are responsible for contagious zoonotic infections in humans(1).

The poxviruses have a very complex structure, which is distinct from other viruses. The virus particle size is between 200 to 400 nm in length and has a brick-shaped or ovoid morphology, which is composed of a core containing a double-stranded DNA genome (ranging from 127 kb to 456 kb encoding for hundreds of proteins), surrounded by lateral bodies and an outer envelope. The complex structure and the complex genome of the poxvirus lead to significant challenges in fully understanding the functions of their genes and infection mechanisms. The replication of the poxviruses extensively occurs within the cytoplasm (called virosome or viral factory) of host cells, although other DNA viruses mostly replicate within the nucleus. The genome of the virus can be divided into three main functional categories: early, intermediate, and late genes, which regulate replication, immune evasion, and structural protein synthesis. There are two major forms of the poxvirus, which take the role in two different infection mechanisms: primary host infections and systemic infections(2).

The recent outbreak of monkeypox drew attention to the study of other pox virus species, which have historically frequently been infecting humans. Among the poxvirus species, monkeypox, cowpox, and Orf poxvirus are major zoonotic. The first two species are from *Orthopoxvirus* genus: species from this genus have been extensively studied because of the high morbidity

and mortality rates of the viruses in humans and domestic animals. Researchers have developed vaccines to eradicate smallpox and other pox viruses within the orthopox virus genus. A number of studies have already proven that the vaccines of a species of *Orthopoxvirus* can provide up to 85% cross-protection from infection by another species within the same genus. The Orf zoonotic pox is from the parapox virus genus, which can infect humans, and even vaccine species for animal health can still infect humans. Currently, an effective vaccine has not been developed for humans yet(3).

The Orf is a neglected zoonotic disease which primarily occurs in goats, sheep, and other small ruminants. Humans who work in the agriculture farms and are in close contact with the infected animals can be infected with this virus. Currently, no human-to-human transmission of the Orf virus has been reported. When this virus infects young ruminants, the fatality rate ranges from 10% to 93%. Generally, when it infects both humans and animals, it can cause localized skin lesions, significant discomfort, and complications in infected organisms. The importance of the diseases was undermined by more severe diseases that were caused by the same family of viruses from *Poxiviridae*, like smallpox and monkeypox. Since it is closely related to the high morbidity and mortality rate of infectious pathogens and it has a history of transmission to humans, it can be assumed that it has the potential for spillover risks(4).

The Orf virus carries a dsDNA genome over 138 kb in size, encoding more than 132 genes. The genome is composed of highly conserved regions responsible for transcription and replication, while the virulence genes are located at both ends of the genome. ORFV contains unique genes in its terminal regions that differ significantly from other poxviruses, contributing to its host range and high infectivity. Orf infection can be diagnosed through clinical characteristics and polymerase chain reaction (PCR). There is no specific antiviral drug or vaccine for Orf virus. The preferred method of disease control is preventing secondary infections caused by other pathogenic bacteria(4).

Orf virus is a high-morbidity virus in its primary hosts. It has been reported in infections in sheep, goats, camels, and wild ruminants. In animals, ORFV causes contagious ecthyma, characterized by pustular and ulcerative lesions around the mouth, nose, and hooves. In humans, the infection presents as localized, self-limiting pustular lesions, primarily on the hands and forearms, which evolve over several weeks before resolving spontaneously. The pathogenesis involves viral entry through skin abrasions, followed by replication in epithelial cells, leading to localized tissue damage, inflammation, and lesion formation. The virus also produces immune-modulating proteins, enabling it to evade host immune responses(4).

The species in the *Poxviridae* family has a very distinct structure compared to another virus which allows them to be exceptionally stable in the environment, spread, and infect humans. Among these pox viruses, Orf virus is noted as the most stable one in the environment, and it can live in a dry place for up to 17 years. The morbidity rate of this virus in the primary host is exceptionally high, despite the infection rate still being limited in humans. The fatality cases caused by this virus in humans have been reported in immune-impaired patients, despite the main cause of death not being approved by this virus infection. According to worldwide research on this virus by genomic characterization pointed out that the evolution rate and emergence of new clades have constantly increased recently. Moreover, there are constantly increases in the population of the primary host of this virus in Thailand and globally that have

been reported in Statista. The rising trend of goat farming may elevate the risk of infection in humans(5).

Additionally, the specific set of proteins, including viral polymerase, attachment, entry, and immunomodulatory proteins of the vaccinia pox and other *Chodorpoxviruses* have been studied extensively. In previous studies, it has been confirmed that the entry fusion complex proteins combine with multiple viral proteins. These proteins also play a role in viral pathogenesis. Some of the proteins have an important function as immunomodulatory proteins, such as E3L in *Orthopoxvirus* and *Parapoxvirus*. E3L is one of the evolutionarily acquired immunomodulatory proteins present in poxviruses and is responsible for conferring resistance to interferons among poxviruses(6).

Like other viruses, the poxviruses are assumed to use specific receptors in humans. However, the specific receptors that have been utilized by the poxvirus have not been revealed yet. The entry fusion complex proteins of the poxviruses are within the central conserved region and show homology within the family. As mentioned above about human infection and the binding ability of Orfv to human skin cells. However, the mechanism and receptor for viral entry need to be explored. E3L is a homologous protein found in both *Orthopoxvirus* and *Parapoxvirus*. This protein might have an important role in immunomodulatory functions in human skin infection of Orf virus. Moreover, polymerase protein is an important protein for viruses that also shares conserved regions and functions and determines viral fitness in the specific host. This protein from the Orf virus might play a significant role in viral infection. It is very important to study the viral-host interaction between the selected key proteins of the virus and human receptor proteins to clearly understand the virus entry step. This information will provide valuable structural information for the future development of antivirals and vaccines development(7).

Sequencing methods are essential to study the genomic alteration in viral protein. The genomic alteration in key genes of the Orfv strains might affect the transmission efficiency of this virus to humans. Molecular docking is the application of computational techniques to identify the interactions between two molecules. In drug discovery, the technique has already been widely used to elucidate potent drugs by predicting binding scores and patterns of molecules. There are two major steps in molecular docking: sampling and scoring. First, it generates possible binding sites for the two molecules, followed by scoring based on conformation-dependent binding affinity. Researchers have already utilized these algorithms to comprehend viral-host interactions. Understanding the mechanisms of viral-host interactions is crucial for developing effective treatments and preventive measures(8).

Although Orf virus has spillover potential, being related to other poxviruses, research on human infections is rare. There is no genomic data about the Orf virus in Thailand. As a result, the interactions at the molecular level are not yet fully understood. In this research, we are going to extensively apply genomic characterization and molecular docking to study the interactions between the key proteins of the Orf virus and human receptors. The results will increase knowledge of the transmission of the orf virus to humans and provide useful data by predicting potential emerging risks.

Hypothesis

1. Genetics of Thai isolated Orf virus difference from other countries.
2. The entry proteins and immunomodulatory proteins of the Orf virus involved in transmission and human infections by interaction with the host receptor proteins

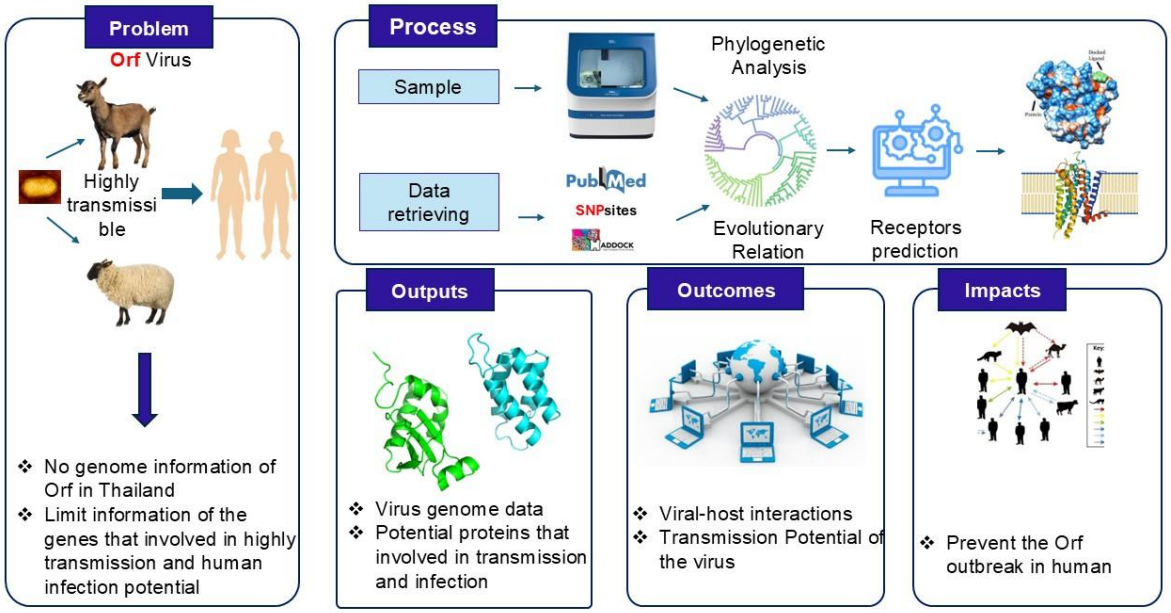
Research questions

1. Are there any Orf circulates in Thailand?
2. Are there any differences in Thai's Orf compared to other Orf strains?
3. What is the evolutionary relation of the Thai isolated Orf virus to the other public strains?
4. What potential Orf proteins can bind to the published host receptors or have immunomodulatory interactions?
5. Does the binding efficacy of the virus and receptor affect the transmission efficiency of the virus to humans?

Objectives of the study

1. Extraction and identification of the Orf virus from the Goat samples.
2. Phylogenetic analysis of the Orf virus from the database and the samples
3. Select the proteins that may involve in transmission to humans by literature review and genetic analysis
4. Study the binding potential of the selected proteins of the Orf pox virus with the host receptors

Conceptual framework



2 Method

2.1 Selection of the interested proteins

2.1.1 Retrieving of the genomic sequence data

The genomic nucleic acid sequences data of the orf virus were retrieved from National Center for Biotechnology Information(NCBI, www.ncbi.nlm.nih.gov/) on 13 May 2025. The detailed information about host species, the country of origin and the year of detection are listed in Table 1. The genomic sequences which did not provide information of host, country and collection date were removed from downstream analysis.

2.1.2 Gene prediction and annotation

To avoid different genomic annotation methods leading to deviation in subsequent analysis, all genome sequences were reannotated using Prokka v1.14.6 in Galaxy Europe(usegalaxy.eu) uniformly using the same parameters (settings:--kingdom Viruses, remaining settings: default). Functional annotation and protein classification were conducted through sequence comparison using BLAST against the Nucleo-Cytoplasmic Virus Orthologous Groups (NCVOGs) dataset (https://www.ncbi.nlm.nih.gov/research/cog-project/). The ORFV coding sequence files generated by Prokka (suffixed with .faa) were used to identify orthologous groups with Orthofinder v2.5.2.

2.1.3 SNP analysis

The single-copy core genes were identified from 43 Orf pox complete genome sequences using Orthofinder v2.5.2. Then, each Orf single-copy core gene's sequences were aligned using MAFFT v7.487 with default parameters. The SNPs of each orf single-copy core gene were identified using SNP-sites v2.5.1. The comparison of SNP numbers and average SNP density across the selected genes were performed by using the ggpubr R package.

2.1.4 Nucleotide and amino acid level similarity

To see the amino acid level similarity of the membrane between the Orf pox virus and the other two reference pox virus vaccinia and Monkeypox viruses, we performed blastp of the total 14 membrane protein of the Orf pox virus.

2.2 Machine Learning Based Prediction of Viral Receptor Usage

A model for prediction of the potential receptor usage of the virus will be established, especially for the Poxviruses including the Orf virus. The model will be trained by using the experimentally validated virus–receptor interaction pairs from skin-infecting viruses. And then apply the model for ORFV attachment proteins, and attachment proteins from other poxviruses.

2.2.1 Viral attachment protein selection

Viral proteins caused the skin infection in humans were included if they are experimentally reported or strongly inferred to mediate viral attachment or entry contain extracellular domains exposed to host cells, Have full-length and high-quality protein sequences available. Protein sequences were retrieved from UniProtKB and cross-validated using NCBI RefSeq.

2.2.2 Human receptor dataset

Human receptors were included if there is experimental evidence for direct viral binding, they are cell surface or membrane-associated proteins and they are expressed in skin-related tissues (keratinocytes, epidermis). Receptor sequences of human were obtained from UniProtKB and Expression confirmed via Human Protein Atlas.

2.2.3 Virus–receptor interaction pairs and model development

The positive interaction pairs will be defined as the pair protein of virus and human receptor and they were previously confirmed by the experimental method. The negative pairs will be constructed by using pairs not related to the viral attachment and entry, and the pair will also be confirmed by protein interaction analysis in STRINGdatabase to select only there is no know interactions. Additionally, compartment-based exclusion by using UniProt subcellular localization data, viral proteins known to be extracellular will be paired with human proteins strictly localized to incompatible compartments (e.g., nucleus, mitochondrion). The final negative dataset was balanced 1:1 with the positive dataset. Similar to the previous study, Sequence-level features, Structural features, Functional features, Host receptor features, Virus–receptor pairwise features will be used in the model development.

For confirmation of the protein-protein interactions, the pairs will be compared to the PPIs in P-HIPSTer. The viruses which have the experimental evidence and the viruses which showed the strong possibility to infect the human skin according to data from Human Viral Database will be selected as a successful candidate vector in training data. The predicted RBP and human receptor pairs will be ranked by the score provided by the selected model.To validate the accuracy of the constructed model, the vectors with 20% of the test data will be used. The RBPs and receptors pair showing more than 60% of test data within the top 3, the model will be selected for prediction of ORFV and other poxviruses receptor. The prediction accuracy of the established will be compared to the previous develop model by Zheng 2020.

2.3 Species identification of the Orf virus

2.3.1 Sampling

Sampling will be performed between March 2024 and December 2025 in the Khon Kaen district goats' herd. At the farm, newborn goats(kids), and adult animals showed clinical signs of ORFV infection, that will be also confirmed by molecular analyses. The orf virus positive samples involved in our research during their diagnostic activity were previously confirmed by virome sequencing. This sampling will not provide epidemiological indications regarding the incidence and geographical distribution of ORFV on this region.

2.3.2 Nucleic acid extraction, PCR and Sequencing

DNA extraction from the collected blood and milk samples was performed according to extraction kit protocol. The semi-nested PCR technique was carried out on DNA from all samples using the PP1, PP4 and PP3 oligonucleotide primers, which amplify sequences within the B2L gene encoding the major envelope protein. The sequences of PPP-1, PPP-4 (594-bp) and PPP-3 (235-bp) were 5′gtc gtc cac gat gag cag ct-3′, 5′-tac gtg gga agc gcc tcg ct-3′ and 5′-gcg agt ccg aga aga ata cg-3′, respectively. These oligonucleotide Primers are widely used for molecular identification and phylogenetic analysis of the ORFV (parapoxvirus) (Inoshima, Morooka, and Sentsui 2000). The PCR reactions were performed in two steps. For the first step, 2 µL of DNA was subjected to thermocycling in a 25-µL reaction mixture containing 2.5U of Taq DNA Polymerase (Transgenbiotech, China), 2.5mM dNTP mix, 10 pmol of each oligo nucleotide primers(PP1 and PP4), and 10X PCR buffer. The thermal cycling conditions were as follows: initial denaturation at 94◦C for 5 min, followed by 35 cycles of denaturation at 94◦C for 30s, annealing at 55◦C for 20s and extension at 72◦C for 30s. A final extension step was performed at 72◦C for 10 min. Semi-nested PCR was then performed using 2 µL of the first PCR product with the PPP-3 and PPP-4 oligonucleotide primers under the same conditions.

The PCR products were visualized on a transilluminator after electrophoresis in a 1% agarose gel containing Safe-Red DNA stain (Vivantis, China). Approximately 21 ul of amplicon remaining from samples detected positive by gel electrophoresis was used for sequence analysis. The PCR products were sequenced by a commercial company (U2bio, Thailand) automatic sequence analyser (ABI, USA).

3 Results

3.1 QC and basic characteristics

As of 13 May, 2025, 53 Orf pox whole genome sequences have been published, of which 43 were selected through the genomic information of country, collection date and host. The genetic map illustrating genome length, core and variation across region revealed that genome length ranged from 121,730bp to 140707.

Table1: List of WGS sequences

Accession	Species	Length	Country	Host	Collection_Date
PV126639.2	Orf virus	138500	China	Ovis aries	2024
PQ685033.1	Orf virus	126218	Morocco	Ovis aries	2020
PQ374835.1	Orf virus	130535	China	Ovis aries	2024
PQ374836.1	Orf virus	130583	China	Ovis aries	2024

PP943425.1	Orf virus	127637	China	Capra hircus	1/31/2012
PP943426.1	Orf virus	129459	China	Capra hircus	1/31/2012
PP943427.1	Orf virus	127372	China	Capra hircus	1/31/2012
OR637323.1	Orf virus	129588	Cuba	Ovis aries	10/10/2008
OR637324.1	Orf virus	121730	Cuba	Ovis aries	12/25/2008
OR637325.1	Orf virus	124146	Cuba	Capra hircus	11/10/2007
OR427036.1	Orf virus	134041	Rwanda	Syncerus caffer	10/13/2015
OR453678.1	Orf virus	134041	Rwanda	Syncerus caffer	10/13/2015
ON805830.1	Orf virus	137160	Argentina	Ovis aries	2018
ON805831.1	Orf virus	137340	Argentina	Ovis aries	2018
ON805832.1	Orf virus	137214	Spain	Ovis aries	2018
ON805833.1	Orf virus	137891	Spain	Ovis aries	2018
ON380499.1	Orf virus	140179	India	Capra hircus	2005
ON380500.1	Orf virus	139966	India	Capra hircus	2005
OP562382.1	Orf virus	131558	Malaysia	Capra hircus	2019
OP151442.1	Orf virus	127160	China	Capra hircus	10/1/2020
ON932451.1	Orf virus	140707	China	Ovis aries	2019
ON691519.1	Orf virus	128110	Italy	Ovis aries	2/13/2020
ON691520.1	Orf virus	128257	Italy	Ovis aries	3/10/2020
ON691521.1	Orf virus	129776	Italy	Capra hircus	4/20/2020
ON691522.1	Orf virus	131660	Italy	Ovis aries	3/26/2021
ON691523.1	Orf virus	129506	Italy	Ovis aries	5/24/2017
ON691524.1	Orf virus	129858	Italy	Ovis aries	10/11/2019
ON691525.1	Orf virus	130411	Italy	Capra hircus	7/3/2019
MW537048.1	Orf virus	132124	Malaysia	Capra hircus	2018
MN648218.1	Orf virus	138446	China	Capra hircus	6/10/2018
MN648219.1	Orf virus	138495	China	Ovis aries	5/15/2018
MT332357.1	Orf virus	139807	India	Capra hircus	1/21/2017
MN454854.1	Orf virus	134893	USA	Ovis aries	2019-01
MG712417.1	Orf virus	140413	China	Ovis aries	11/16/2016
MG674916.2	Orf virus	139287	China	Capra hircus	12/2/2016
KY053526.1	Orf virus	136643	China	Ovis aries	2012-01
KP010353.1	Orf virus	138231	China	Capra hircus	10/4/2012
KP010354.1	Orf virus	139866	China	Capra hircus	2/20/2012
KP010355.1	Orf virus	132111	China	Capra hircus	12/30/2011
KP010356.1	Orf virus	139112	China	Capra hircus	4/22/2012
KF234407.1	Orf virus	137080	China	Ovis aries	10/26/2011
KF837136.1	Orf virus	134104	Germany	Homo sapiens	1996
LR594616.1	Orf virus	132823	France	Homo sapiens	2017-09

3.2 Gene prediction and annotation

From the prokka analysis, the coding genes of the ORFV genomes were identified by using reference sequence Orf pox virus. The location of the core region in all the Orf pox virus genome in this study was found to be between ORF009 and ORF111. The flanking core regions are designated as variable regions. Genes responsible for DNA replication, recombination, and repair, as well as virion structure and morphogenesis, are generally located in the conserved

regions, while genes related to host-virus interactions are generally located in the variable regions.

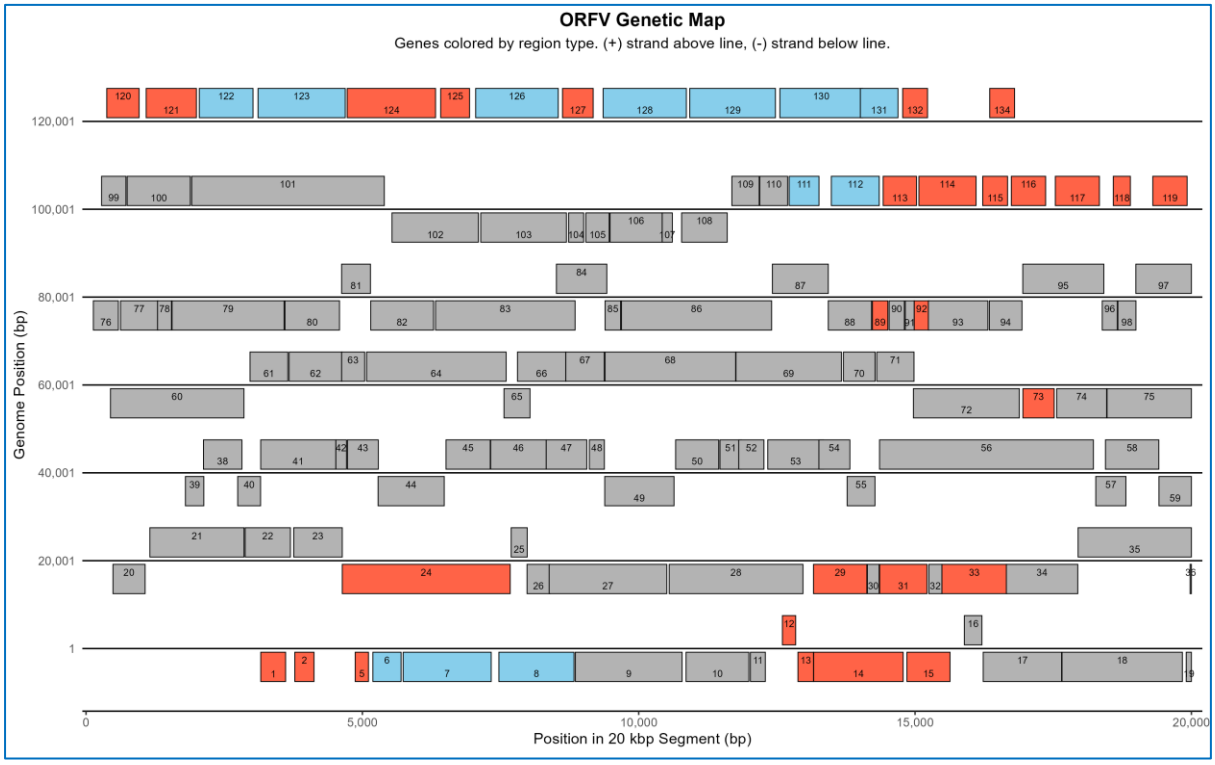


Figure 1: Gene Map of the Orf pox virus. Unique gene of the Orf pox are shown in red color. Blue represents the genes in variable regions

3.3 SNP detection

A total of 132 coding genes with significant similarity to reference sequences were identified from 43 Orf genome sequences. SNP detection was subsequently performed on each of the 132 CDS. The average SNPs density of the envelope proteins and immunomodulatory proteins orf pox virus were analysed by using SNP sites. We found that the average SNP density in variable regions is higher than in core regions. The results of statistical analysis showed that although there was no significant difference in SNP number, the average SNP density in variable regions was slightly higher than that in core regions. The gene with the highest SNP density was ORF131 and ORF112 located in variable regions.

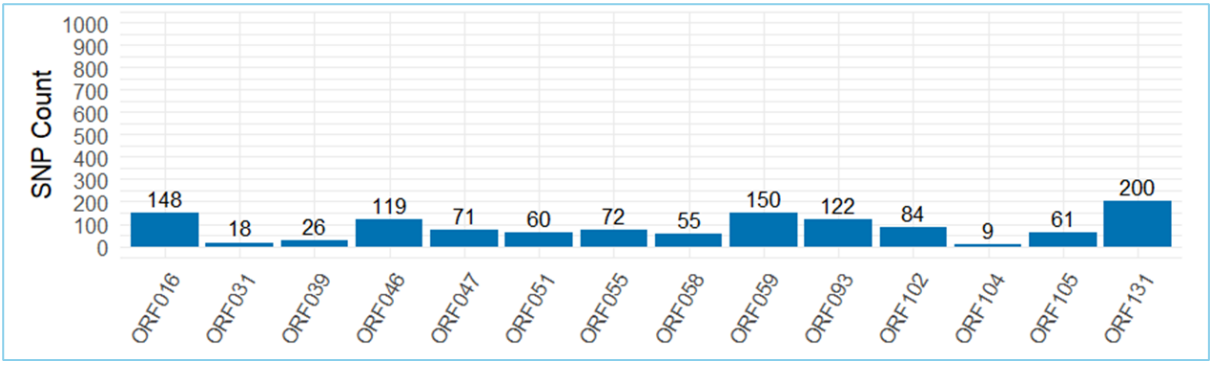


Figure 2: SNP density of membrane coding sequence of the Orf pox virus

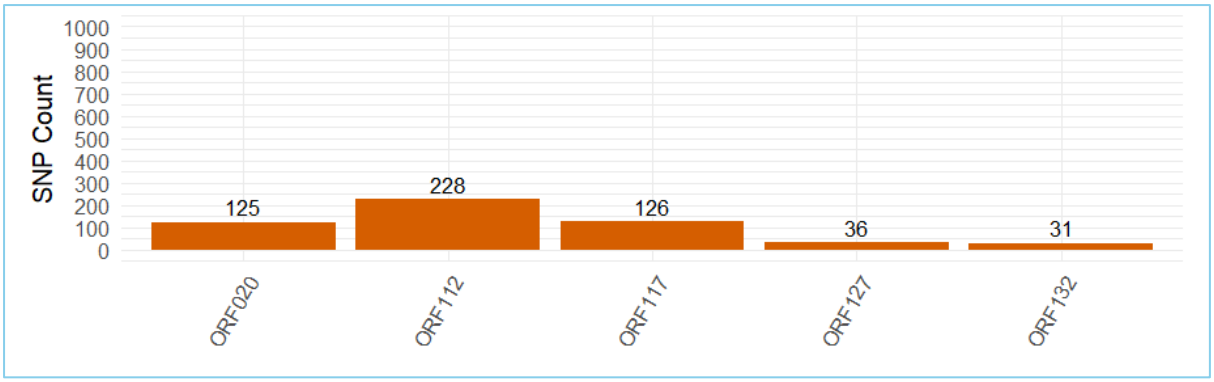


Figure 3: SNP density of Immunomodulatory coding sequence of the Orf pox virus

3.4 Nucleotide and amino acid level similarity

In further analysis, we analyze the amino acid level similarity of the membrane proteins between the Orf pox and other two reference pox virus's species by using blastp of NCBI. We can identify that that the ORF047 and ORF055 showed the highest significant similarities ratio to the reference pox virus membrane proteins.

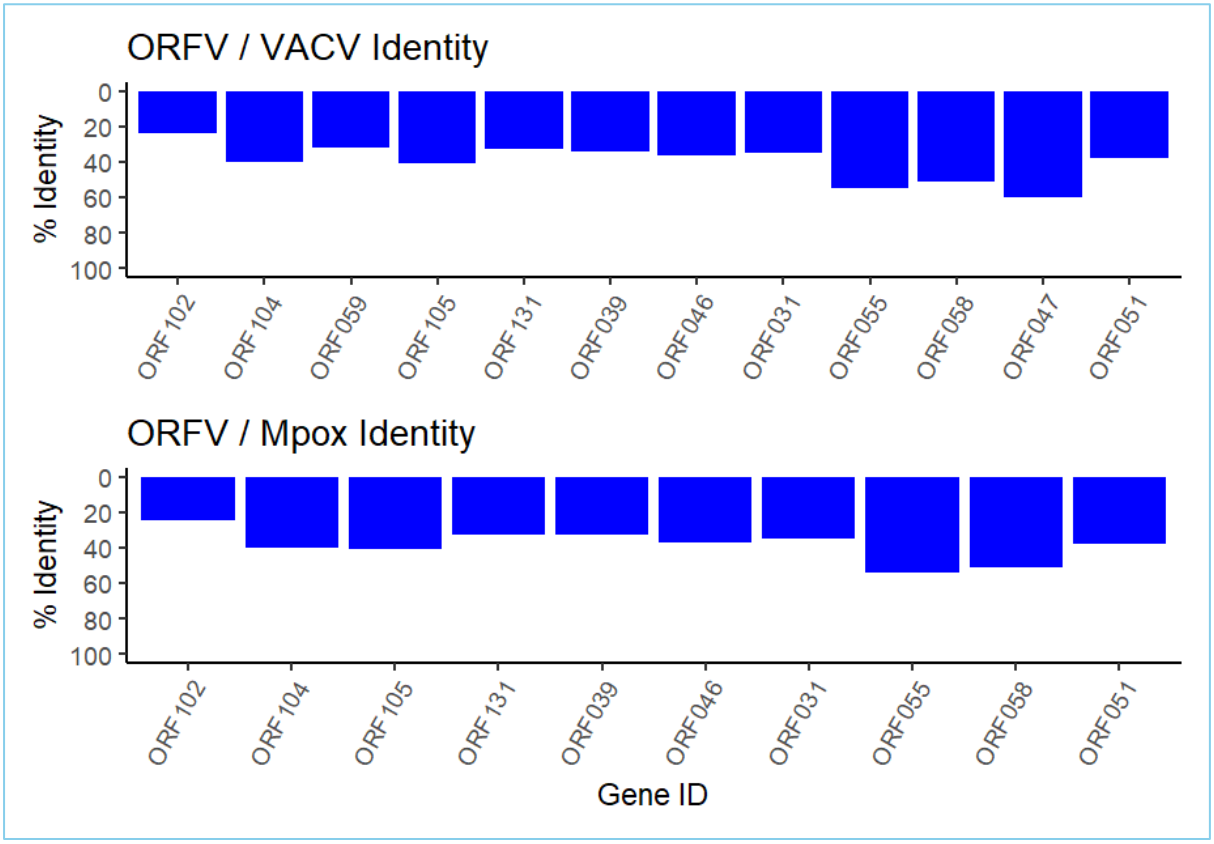
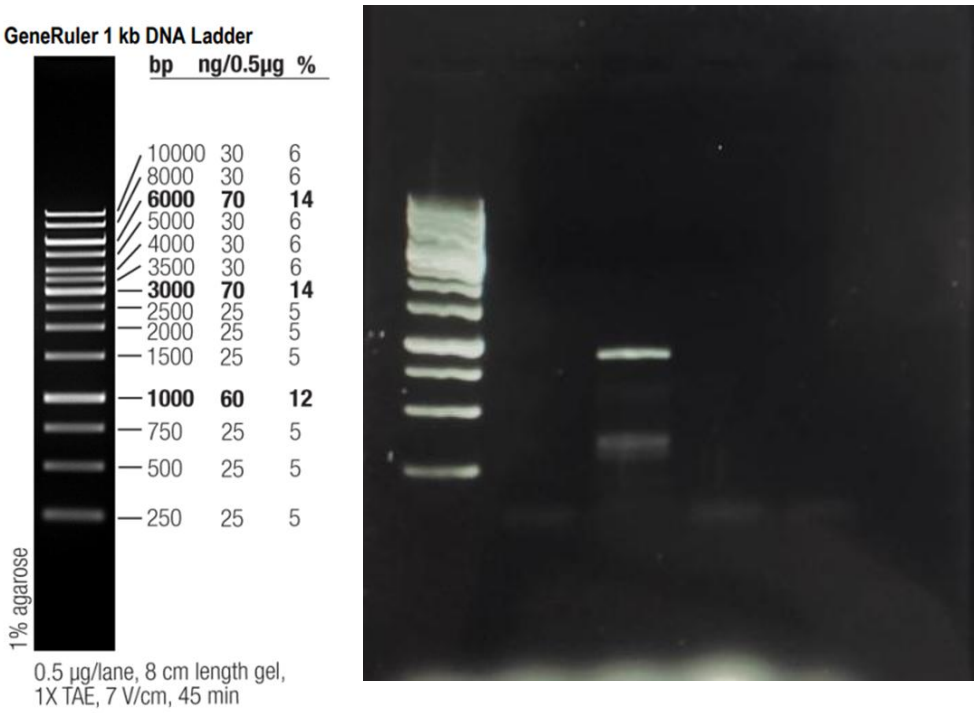


Figure 4: The significant similarities ratio between the Orf pox virus and other two reference pox virus species (VACV and Mpox)

3.5 PCR, Gel electrophoresis and Sequencing

The potentially positive PCR bands to the Orf virus were detected around 300bp and 1000bp. The band positions are higher than the expected location of 593bp and 235bp. After the gel extraction, the nucleic acid composition of the positive bands was detected by using Nanodrop and it have 36ng/ul and 6.6ng/ul respectively.



Discussion

In this study, set a criteria to construct a receptor prediction model for supporting of the further analysis. In the PCR confirmation of the Orf virus positive suspected sample. the PCR bands were detected in the unspecific position of the B2L gene with a high intensity. To confirm this is the actual Orf virus, further confirmation by sequencing are required.

Reference

1. Fenners Veterinary Virology (N James MacLachlan, Edward J. Dubovi).
2. Upton C, Slack S, Hunter AL, Ehlers A, Roper RL. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol. 2003;77(13):7590-600.
3. Yu Z, Zou X, Deng Z, Zhao M, Gu C, Fu L, et al. Genome analysis of the mpox (formerly monkeypox) virus and characterization of core/variable regions. Genomics. 2024;116(1):110763.
4. Kassa T. A Review on Human Orf: A Neglected Viral Zoonosis. Res Rep Trop Med. 2021;12:153-72.
5. Alajlan AM, Alsubeeh NA. Orf (Ecthyma Contagiosum) Transmitted from a Camel to a Human: A Case Report. Am J Case Rep. 2020;21:e927579.
6. Nemeth C, Boros A, Meszaros E, Gyomorei C, Albert E, Pankovics P, et al. Human orf virus (family Poxviridae) infection following a lamb bite in Hungary. Arch Virol. 2024;169(3):59.
7. Srinivasan Rajsri K, Rao M. Poxvirus-driven human diseases and emerging therapeutics. Ther Adv Infect Dis. 2022;9:20499361221136751.
8. Coradduzza E, Sanna D, Scarpa F, Azzena I, Fiori MS, Scivoli R, et al. A Deeper Insight into Evolutionary Patterns and Phylogenetic History of ORF Virus through the Whole Genome Sequencing of the First Italian Strains. Viruses. 2022;14(7).