Genomics-Informed Surveillance for Elimination of Rabies in the Philippines

by
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Abstract

Surveillance comprises the collection, analysis and dissemination of health-related data to support planning, implementation and evaluation of public health response. Genomic surveillance involves incorporating genomic sequencing to understand transmission dynamics and the spread of outbreaks, thereby complementing traditional surveillance. The utility of genomic sequencing for supporting rabies elimination is demonstrated by its capacity to track viral sources, investigate host shifts, and identify cross-border incursions. However, despite its potential, genomic surveillance remains underutilised and its integration into public health interventions has been limited, particularly in countries where rabies is endemic.

In the Philippines, rabies remains a significant public health threat, with hundreds of human deaths annually and widespread canine rabies transmission across all island groups. While national programs have made progress in dog vaccination and awareness campaigns, surveillance continues to rely heavily on traditional epidemiological data, with limited integration of molecular or genomic approaches. Prior to this thesis, the application of whole genome sequencing to rabies virus surveillance in the Philippines was minimal, and no standardized genomic framework existed to support outbreak investigations or inform control strategies.

This thesis explores the integration of genomic data into epidemiological surveillance in rabies control efforts in the Philippines, with the ultimate goal of contributing to the global "Zero by 30" campaign to eliminate human deaths from dog-mediated rabies. Using nanopore sequencing technology, I tested and validated a cost-effective whole genome sequencing workflow from sample-to-sequence-to-interpretation. The workflow was tailored to meet the demands of Low- and Middle-Income Countries (LMIC) settings, providing a scalable model for genomic surveillance. Using this approach, I generated whole genome sequences from rabies samples collected across different regions of the Philippines. For phylogenetic inference, I used BEAST and auxiliary programs (Beauti, Tracer, LogCombiner, TreeAnnotator), and accounted for sampling bias by employing for Bayesian Tip Association Statistics Testing to assess strength of association. In addition, using epidemiological data and samples from an outbreak in Romblon, I reconstructed the likely source and timing of viral incursion(s).

Phylogenetic analysis of the generated sequences, alongside publicly available genomes, revealed the presence of four major rabies virus clades in the Philippines, corresponding to the three main island groups Luzon, Visayas and Mindanao and an older lineage within Luzon, estimated to have emerged around 1970. This lineage, predominantly found in Calabarzon, aligns with previous studies showing the region as the source of the most recent common ancestor (MRCA) of circulating strains. A strong geographic association of rabies virus (RABV) was evident at the island and regional levels, though less pronounced at finer geographic scales. Luzon exhibited the highest genetic diversity, likely due to better sampling coverage. In contrast, the Visayas had fewer samples, contributing to reduced statistical support in this region.

By combining genomic data and epidemiological data, this thesis established a practical approach for enhanced rabies outbreak detection and response. These findings highlight how genomic surveillance can support region-specific strategies and improve rabies control across diverse epidemiological settings. The development of a rapid, cost-effective sequencing workflow contributes to overcoming existing barriers related to cost, infrastructure, and expertise.

My findings have significant implications for rabies elimination and reinforce the importance of integrating genomics into the national surveillance systems. They also underscore the need for a One Health approach through intersectoral Bite Case Management (IBCM). Future research directions should focus on expanding genomic databases, performing finer-scale phylogeographic analyses, and addressing socio-cultural factors affecting control efforts. Ultimately, the tools and insights from this thesis can advance not only rabies control but also the broader application of genomic surveillance for other zoonotic and infectious diseases in the region.

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I dedicate this thesis to my parents, Cleto and Rosalily Bautista.

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Author's Declaration

I declare that the contents presented in this thesis are the result of original research conducted by the author, Criselda Bautista, except where otherwise stated. Where work has been derived from collaboration with others, my personal contribution to each chapter is detailed below. Chapters 2 and 3 were produced as stand-alone manuscripts that have been published, while Chapter 5 is currently under revision following an invitation from the journal to revise and resubmit. These three chapters were all produced in co-authorship with my supervisors and a wider research project team. My personal contribution is as follows:

Chapter 1: I am the sole author.

Chapter 2: *Published as:* Jaswant G*, Bautista CT*, Ogoti B, Changalucha J, Oyugi JO, Campbell K, Mutunga M, Thumbi SM, Hampson K*, Brunker K*. Viral sequencing to inform the global elimination of dog-mediated rabies - a systematic review. One Health Implement Res 2024; 4:15-37. http://dx.doi.org/10.20517/ohir.2023.61. *Denotes equal contributions. CB and GJ conducted the literature review, data analysis and initial manuscript drafting. CB was responsible for screening, summarizing, and extracting data from journal articles representing Asia and contributed to the extraction and summarizing of data from some of the journal publications from Latin America. CB also consolidated the extracted data and co-wrote the results section as well as the discussion part for the Asia region and partly for Latin America. KH and KB provided critical review and final manuscript edits.

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Chapter 4: *In preparation for submission as:* Bautista C., Katie Hampson, Kirstyn Brunker. Characterization of the diversity and distribution of RABVs in the Philippines. Molecular work and sequencing done by CB. Phylogenetic analysis done by CB with supervision from KB. CB drafted the manuscript which was edited by KB and KH.

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Chapter 6: I solely wrote the final discussion chapter.

Criselda Bautista

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Chapter 1

Overview

1.1 Genomic surveillance

Disease surveillance, a critical component of public health, involves collecting, analysing, and interpreting data to plan, implement, and evaluate public health policies (Osterholm & Hedberg, 2015). The core functions of surveillance include case detection, data sharing, analysis, informing control measures and their implementation, as well as monitoring and evaluation to guide public health responses (WHO, 2006). Effective communication is crucial for disease surveillance in enabling efficient response measures like contact tracing and vaccination campaigns, while also raising awareness and promoting community engagement through positive behaviour change towards health seeking behaviour and responsible pet ownership (Aranda et al., 2022).

Pathogen genomic epidemiology can inform public health by providing detailed insights into disease dynamics. This field integrates pathogen genomic and epidemiological data using computational approaches to provide a detailed understanding of disease dynamics (Gardy & Loman, 2018). In the control and management of infectious diseases, combining pathogen genomic data with traditional epidemiological methods can inform more targeted and effective health interventions and strategies. As genomic technologies advance, genomic surveillance has the potential to improve public health practices in outbreak response, and control (WHO, 2022).

More specifically, genomic surveillance enhances traditional surveillance by using pathogen genomic data to track the spread, evolution, and transmission dynamics of diseases at a molecular level (Armstrong et al., 2019). Genomic surveillance can be conducted through partial genome or whole genome sequencing (WGS). Partial genome sequencing focuses on specific regions of the viral genome, such as those that vary significantly between virus variants and lineages, making it faster and potentially more cost-effective. This approach is useful for tracking viral diversity and identifying circulating variants. However, partial genome sequencing may miss mutations outside the targeted regions, limiting its ability to provide a complete evolutionary picture. Whole genome sequencing, on the other hand, analyses the entire genome, offering a more comprehensive understanding of the virus. WGS allows for more detailed phylogenetic analysis, greater resolution in distinguishing between closely related viruses, and the identification of mutations that may impact virulence or transmission (Brunker et al., 2015). While WGS requires more resources, it offers a more comprehensive understanding of rabies virus evolution and spread. In a recent study it was demonstrated how combining partial and whole genome sequences allows for precise dating

and confident estimates of historical RABV spread between countries (Holtz et al., 2023). This approach minimizes geographic and genetic biases associated with focusing on specific genes, providing a more accurate and comprehensive view of genetic diversity and spread.

Public health genomics has become an increasingly powerful tool for disease surveillance, providing crucial insights into pathogen evolution, virulence, and transmission (Bianconi et al., 2023; Pronyk et al., 2023). The World Health Organization (WHO) has developed a global genomic surveillance strategy for pathogens that aims to link and embed pathogen monitoring (genomic surveillance) into broader, more traditional standard surveillance systems, strengthen capacities, and unite stakeholders under a common vision (WHO, 2022). Enhancing surveillance through the incorporation of pathogen genomic data in addition to epidemiological data is widely used in other disease and pathogen surveillance systems like for COVID-19 (Nextstrain) (Hadfield et al., 2018), Influenza (Global Influenza Surveillance and Response System) (*Global Influenza Surveillance and Response System (GISRS)*, 2024), Malaria (MalariaGEN) (*MalariaGEN*, 2024), and Antimicrobial resistance (Global Antimicrobial Resistance Surveillance System) (*Global Antimicrobial Resistance and Use Surveillance System (GLASS)*, 2024).

1.2 Rabies surveillance

Rabies is an acute fatal infection of the central nervous system, leading to severe illness and death in both humans and animals. Once clinical symptoms manifest, the outcome is nearly always fatal (Rupprecht et al., 2002). The disease is primarily transmitted by mammals, particularly those belonging to the Carnivora and Chiroptera orders (Brunker et al., 2020). Among these hosts, dogs are the major reservoir and vector responsible for the majority of human deaths (Rupprecht et al., 2002). Other reservoirs include wild canids such as coyotes, jackals, and raccoon dogs (Brunker et al., 2020). Certain rabies variants show host specificity, such as variants found in raccoons (Szanto et al., 2011), mongoose (Davis et al., 2007), and ferret badgers (Miao et al., 2022; Ming et al., 2018). The rabies virus (RABV) is a bullet-shaped negative-sense, unsegmented, enveloped RNA virus of the genus Lyssavirus. RABV is a member of the Rhabdoviridae family and has been widely studied due to its global distribution and significant impact on public health (Davis et al., 2015). Rabies is a vaccine-preventable disease, yet it causes approximately 59,000 human fatalities annually, primarily due to dog bites (Hampson et al., 2015).

Efforts to control and eliminate rabies have intensified over the years leading to the initiative between the World Health Organization (WHO), the World Organisation for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO) and the Global Alliance for Rabies Control (GARC) to develop a comprehensive strategic plan to achieve zero human deaths from dog-mediated rabies by 2030, also known as "Zero by 30" (WHO/FAO/OIE/GARC, 2018). A coalition of partners, under the umbrella of the United Against Rabies forum (UAR) leverages existing tools and expertise in a coordinated way to empower, engage and enable countries to save human lives from rabies as part of "Zero by 30". Launched since 2018, the "Zero by 30" plan promotes mass dog vaccination campaigns, improved access to post-exposure prophylaxis (PEP) for humans and public education to reduce the burden of rabies in endemic regions. Key strategies within the "Zero by 30" plan include targeting 70% vaccination coverage in dog (primary reservoir) populations, and enhancing surveillance systems to monitor the spread and control of the disease (United Against Rabies, 2020)

The surveillance of zoonoses like rabies is particularly challenging since it requires collaboration between both human and animal health sectors. This entails a "One Health" approach, which is an integrated, unifying approach that aims to sustainably manage the health of people, animals and ecosystems. One Health recognizes the health of humans, domestic and wild animals, plants, and the wider environment are closely linked and interdependent (WHO, 2017b). Moreover, in rabies, genomic investigation studies have been conducted in endemic countries but the full potential of genetic data use in rabies surveillance is yet to be explored. Scaling up rabies sequencing in endemic countries, with laboratory networking could enhance risk assessment and control strategies (Jaswant et al., 2024).

1.2.1 Rabies surveillance in the Philippines

Rabies remains a critical public health issue in the Philippines, with a nearly 100% fatality rate despite being entirely vaccine preventable. Children under 15 account for at least one-third of rabies-related deaths. While the number of human deaths has fluctuated over the years, significant gaps in prevention and control efforts persist. Addressing these gaps is further complicated by the Philippines' unique geography, which poses significant logistical and operational challenges for implementing effective rabies surveillance and control measures. The Philippines with a population of approximately 113.86 million is an

archipelagic country consisting of thousands of islands grouped into three major island groups; Luzon, Visayas, and Mindanao (Philippine Statistics Authority, 2024).

The physical geography of the Philippines, characterized by vast bodies of water separating its islands, hinders the movement of resources, personnel, and information necessary for effective surveillance. The administrative structure, with its decentralized governance, further complicates coordination among local government units (LGUs), regional health offices, and national agencies. Surveillance efforts are further constrained by inadequate human, material, and financial resources. Many areas face political and logistical obstacles that limit the implementation of robust rabies control measures. Limited diagnostic capacity is evident in infectious disease surveillance, particularly in rabies surveillance in the Philippines. Additionally, most epidemiological forms in regional laboratories are still paper based, leading to reporting delays and errors. The lack of systematic data management exacerbates these issues, resulting in fragmented and incomplete data. This limits the ability to accurately monitor rabies trends, trace outbreaks, and plan interventions. These systematic challenges highlight the critical need for innovative approaches, such as integrated bite case management, genomic surveillance, rapid diagnostic test kits to improve rabies control and prevention in the Philippines.

1.2.2 National strategic plan for rabies control

The National Rabies Prevention and Control Committee (NRPCC) was established in 1991 to oversee rabies control efforts in the Philippines. However, early initiatives were hindered by inadequate funding and the lack of a dedicated budget for national and local programs. In 2003, an administrative order was issued, outlining policies for declaring rabies-free zones under the guidance of the National Rabies Prevention and Control Program (NRPCP). The administrative order established specific criteria for rabies-free declarations, including: 1) the absence of locally acquired cases in humans or animals for two years, 2) a well-established surveillance system, 3) enforcement of rabies prevention regulations, and 4) no imported cases in carnivores outside quarantine stations for six months.

To maintain rabies-free status, several key measures were mandated: 1) mass dog vaccination campaigns every three years, with puppies vaccinated at three months and given annual boosters, 2) prophylactic immunization for high-risk individuals such as veterinarians and animal handlers, 3) enforcement of dog control measures, 4) quarantine and movement control of dogs per Department of Agriculture-Bureau of Animal Industry (DA-BAI)

regulations, and 5) continuous information, education, and communication campaigns (The Philippine Government Administrative Order, 2003). This administrative order prompted local governments to strengthen rabies surveillance, enforce dog control policies, and conduct mass vaccinations in pursuit of rabies-free status.

In 2007, Republic Act No. 9482 (Anti-Rabies Act of 2007) formally established the NRPCP as an intersectoral initiative aimed at strengthening rabies prevention and control. The program is implemented by the NRPCC, which includes representatives from government agencies, local government units (LGUs), NGOs, and academic institutions. That same year, the Bohol Rabies Prevention and Elimination Project (BRPEP) was launched, serving as a successful model of rabies elimination. Within 18 months, human rabies deaths in Bohol dropped to zero, and from October 2008 to November 2010, no human or animal rabies cases were detected. Effective surveillance and mass dog vaccination campaigns helped contain isolated cases in 2010 and 2011. The project, which cost USD 450,000, was sustained through community registration fees, demonstrating the importance of continuous funding and local engagement (Lapiz et al., 2012).

As a result of these efforts, by 2017, forty-nine municipalities had achieved rabies-free status (Junio, 2017). However, progress has varied across regions due to differences in resources, infrastructure, and prioritization, highlighting the need for sustained support and coordination to achieve nationwide rabies elimination.

In line with the "Zero by 30" global goal of eliminating human rabies and declaring the country rabies-free by 2030, the NRPCP in consultation with partner agencies developed a national strategic framework in 2019 which focuses on key strategies for rabies elimination in dogs and the improvement of governance, service delivery, financing, regulation, information dissemination, and capacity building (Figure **1.1**) (Department of Health & Department of Agriculture Bureau of Animal Industry, 2019). The national strategic plan is a key milestone marking the progress that the Philippines has achieved in the fight against rabies. However, persisting surveillance challenges and the recent effects of the COVID-19 pandemic have reversed the country's progress as evident in the reported increase in human rabies cases from 300 in 2022 to over 350 in 2023 (Aranda et al., 2022). Coordinated implementation of the country's strategic plan is integral to getting back on track towards achieving 'Zero by 30', but there is still suboptimal intersectoral coordination and weak diagnostic and surveillance capacity within the country.

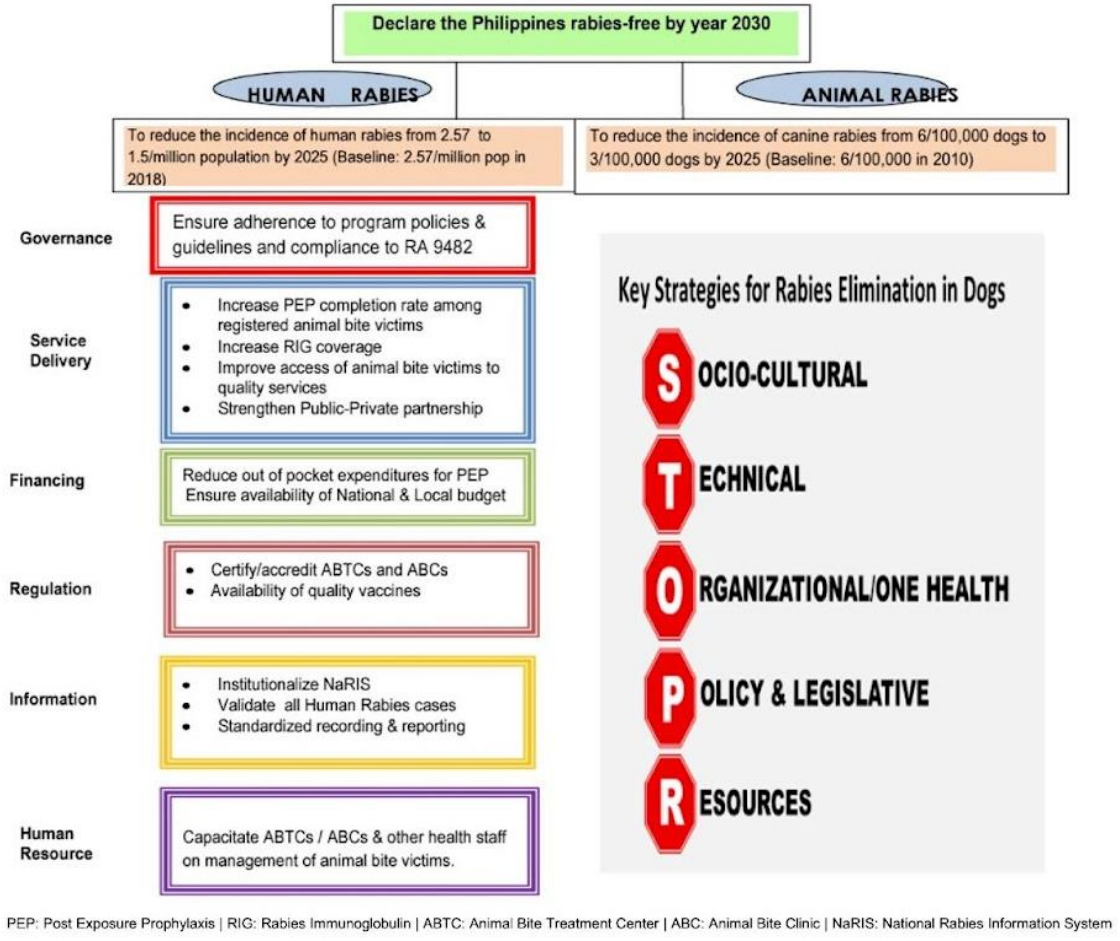

Figure 1.1 **Strategic framework of the National Rabies Prevention and Control Program.** (Department of Health & Department of Agriculture Bureau of Animal Industry, 2019)

1.2.3 Integrated Bite Case Management

Integrated Bite Case Management (IBCM), which has recently been recommended by the WHO as part of the 'Zero by 30' plan, improves rabies control efforts by investigating suspected rabid animals, sharing information with animal and human health investigators for appropriate risk assessments, and ensuring more targeted use of Post-Exposure Prophylaxis (PEP) (WHO, 2018). IBCM has six components: 1) reporting a bite or exposure event, 2) performing a risk assessment, 3) triggering an investigation for any bite deemed high-risk, 4) conducting an animal investigation, 5) observing animal for 10-14 days (to confirm a healthy animal) or collecting samples and diagnostic testing (from dead/euthanized animals), and 6) sharing feedback and investigation results across sectors (WHO, 2018). However, the implementation success of IBCM varies across regions based on local needs and rabies control levels (Figure **1.2**) (Swedberg et al., 2022). Enhancing surveillance with innovative

diagnostic tools like rapid diagnostic tests (RDT) used in the initial screening of animal samples in the field can also strengthen rabies surveillance. Likewise, genomic sequencing of rabies viruses from both human and animal samples is a new technology in the identification of possible origin of cases. Both RDTs and genomic surveillance have potential to further enhance the practice of IBCM and more generally strengthen rabies surveillance in the Philippines.

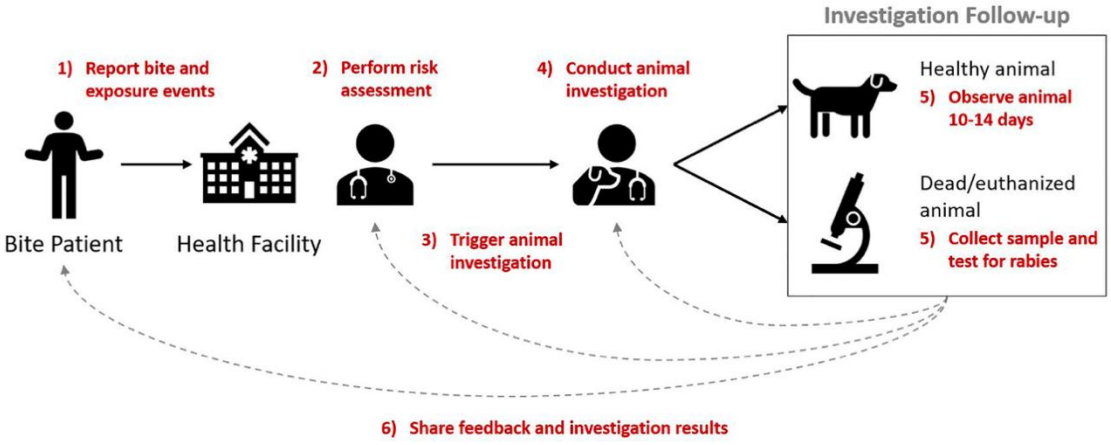

Figure 1.2 **Key components of IBCM.** Six steps of the IBCM process showing how investigation results need to be shared across sectors. Figure reproduced with permission from Swedberg et al., 2022

1.3 Research aims and objectives

The rationale for this thesis is rooted in the need for a more comprehensive approach to rabies control in the Philippines. Gaps in understanding or rabies transmission dynamics have hindered effective control efforts within the country. This research aims to provide a clearer picture of how rabies circulates in the Philippines, including identifying patterns of transmission and factors that influence its persistence by integrating genomic data with epidemiological information. This combined approach will enhance the understanding of rabies dynamics, enabling the development of more effective strategies to control and eventually eliminate the disease in the country. The integration of genomic data with traditional epidemiology provides a strong way to answer key questions about rabies, like how it enters new areas, how it spreads, and what factors affect its transmission. These insights are vital for creating more targeted interventions, using resources more efficiently, and helping achieve the goal of eliminating dog-mediated human rabies by 2030.

This thesis further demonstrates rabies as a model to show how improved genomic surveillance can be applied to other neglected or emerging diseases in the Philippines. By tackling these challenges of genomic surveillance for rabies, it helps to inform strengthened disease surveillance systems and supports the One Health approach, which encourages collaboration across human, animal, and environmental health sectors.

The thesis is composed of six chapters (including this introductory chapter) which are outlined below, including the aim and objective of each of the research chapters.

The aim of Chapter 2 *(Using viral sequencing to improve rabies control: a review)* is to explore how genomic data can guide targeted interventions and improve surveillance efforts for rabies. The objectives of this chapter were to addresses the following key questions: Which genomic sequencing methods are most used in rabies surveillance to date? How has genomic surveillance clarified rabies virus transmission dynamics and the origins and spread of outbreaks? How has genomic data informed dog-mediated rabies control and prevention strategies? What challenges exist in implementing genomic surveillance in low-resource settings? The chapter consists of a systematic review of the role of viral sequencing in eliminating dog rabies in endemic regions, focusing on publications from Asia, Africa, and Latin America. A systematic search of PubMed, Web of Science, and Google Scholar identified original studies published from 2000 to 2023. Overall, this review synthesises the potential of viral genomic data to inform and tailor strategies for controlling dog-mediated rabies in endemic regions.

The aim of Chapter 3 *(Accessible rabies sequencing workflow for low resource settings)* is to present a rapid, affordable sample-to-sequence-to-interpretation workflow for rapid characterization of rabies virus using nanopore technology which is more accessible in Low- and middle-income countries (LMICs). The objective of the chapter is to demonstrate that this workflow is feasible through showcasing worked examples in different settings. Protocols for sample collection, confirmatory diagnosis, and an optimised whole genome sequencing process are presented in detail. A simplified wet lab protocol is tailored to be more cost-effective based on optimised calculations of reagent use. This chapter highlights low-cost library preparation and publicly available bioinformatics pipelines needed prior to phylogenetic analysis. The chapter demonstrates the workflow implementation, emphasising critical steps such as validation of sequencing accuracy and consistency, primer optimization, and the use of negative controls. The chapter also introduces the use of public data and genomic tools for regional and global phylogenetic classification (RABV-GLUE

and MADDOG). Key strengths and challenges of carrying out the whole protocol and the need for specialised training, are discussed. Despite barriers such as limited access to reagents and stable infrastructure, the workflow's portability and affordability make it viable for routine genomic surveillance in LMIC's. Overall, the chapter is written to illustrate the potential of the approach to support rabies control efforts and its transferability to other pathogens offers promising solutions for global disease surveillance.

The aim of Chapter 4 (*Understanding how rabies circulates in the Philippines using whole genome sequencing*), is to provide a more thorough understanding of how rabies circulates across the Philippines and the chapter objectives are to determine when was RABV first introduced into the country and how it has spread between and within regions over time. Following the sample-to-sequence-interpretation workflow described in Chapter 3, I selected rabies positive samples in the Philippines and generated RABV whole genome sequences which were then analysed for evolutionary history and phylogeographic structure using Bayesian Evolutionary Analysis Sampling Trees (BEAST). Using the resulting sequence data, I explore the diversity and distribution of RABV in the Philippines.

The aim of the 5th and final research chapter (*Investigating a rabies outbreak using enhanced surveillance and genomic tools*) is to use an enhanced surveillance approach (IBCM supplemented by genomic surveillance) to investigate a rabies outbreak. Specifically, the objectives for this chapter were to 1) generate RABV whole genome sequences (using the protocol from Chapter 3) from Romblon, a formerly rabies-free province in the MIMAROPA region in the Philippines and 2) analyse these sequences to identify whether the outbreak stemmed from a common source, or from multiple introductions, or from continued undetected circulation in the province. The chapter highlights the use of IBCM and WGS within a One Health framework to track the outbreak. IBCM data on animal cases, human exposures, and deaths were used to track the outbreak with samples collected for subsequent viral sequencing. Using these data this chapter outlines the determination of the outbreak's origin, its spread across the province, and the estimated time of introduction through ancestral character reconstruction.

Finally, in the thesis concluding chapter 6 (*Integrating genomics into rabies surveillance and strengthening One Health in the Philippines*) I discuss the integration of genomics into rabies surveillance based on the learnings from my previous three research chapters. Here, I expound on how IBCM can be instrumental in the improvement of intersectoral coordination during outbreaks in the Philippines and how genomic data can give crucial information on

the transmission dynamics of an outbreak. This chapter explores promising future research directions that have emerged from this thesis, highlighting areas that could enhance the understanding and subsequent control of rabies and other zoonotic diseases. It offers recommendations on the steps needed to fully integrate genomic surveillance into the NRPCP. Additionally, emphasis in the chapter is given to the broader implications for strengthening a One Health approach in the country, which involves collaboration across human, animal and environmental sectors to address public health challenges. These strategies can potentially be extended to the Southeast Asian region, towards a coordinated regional approach to genomic surveillance.

Chapter 2

Viral Sequencing to Inform the Global Elimination of Rabies - A Systematic Review

2.1 Abstract

Background: Rabies is a fatal zoonotic disease, present in almost 150 countries. The ‘Zero by 30’ initiative aims to eliminate human deaths from dog-mediated rabies globally by 2030. This systematic review investigates how viral sequencing can contribute to achieving the 'Zero by 30' goal by improving understanding of viral circulation and the impact of rabies control measures.

Methods: A comprehensive search of bibliographic databases was conducted focusing on research on rabies from regions with endemic dog-mediated rabies published between 2000 and 2023, adhering to PRISMA guidelines. Data were extracted and synthesised to provide recommendations for further research and application to support rabies control.

Results: 220 studies were identified to have documented rabies virus sequences from 94 countries, primarily using first-generation technology to produce partial genomes and with sequencing predominantly conducted overseas rather than in-country. Dogs were identified to be the primary rabies virus reservoir in these regions, although some studies identified more localised wildlife reservoirs. Clade classifications were commonly based on host association or geographical location, however, lack of standardised methods and nomenclature for classifying lineages limited comparison at higher resolution. Cross-species transmission, and both local and long-distance transmission were identified, although quantitative inference was limited. Sequence data was particularly useful for identifying transboundary spread and incursions, investigating host shifts, and tracing sources of human rabies, with endemicity typically characterised by the identification of multiple co-circulating viral lineages.

Conclusion: There is an urgent need for standardised classification methods and phylogeny-based nomenclature for rabies viruses, and for improved sequencing capacity in regions with endemic dog-mediated rabies, including proficiency in bioinformatics and phylogenetics. Our findings emphasise the critical need to foster international cooperation and coordinate rabies control efforts to reduce transboundary spread, limit reintroductions and maintain progress towards the 2030 target.

Keywords: Rabies virus; genetic sequencing; phylogenetic analysis; rabies control and prevention; surveillance

2.2 Introduction

Rabies Virus (RABV) poses a major public health threat, causing around 60,000 deaths annually, almost exclusively in Low- and Middle-Income Countries (LMICs) (Hampson et al., 2015). The virus is most commonly transmitted through bites from infected hosts in the orders Chiroptera and Carnivora (Worsley-Tonks et al., 2020). Domestic dogs are the main source of transmission to humans but, as a multi-host pathogen, wild carnivores also serve as primary RABV hosts with host-associated variants recorded in certain geographies (WHO, 2017a). For example, wildlife such as raccoons, skunks and foxes each maintain different RABV variants in localities across North America (WHO, 2017a). Generally, RABV is referred to according to these host-associated variants (sometimes termed biotype (Sabeta et al., 2018), see defined key terms in Box 1).

Box 1. Definitions of key terms in the context of RABV

Term	Definition
Cross-species transmission	Transmission events from one (host) species to another, that occasionally result in a host shift, whereby a new transmission cycle is established, but more frequently leading to short-lived chains of transmission in a different host or dead-end infections with no onward transmission (Faria et al., 2013; Viana et al., 2014).
Reservoir	One or more epidemiologically connected populations in which the pathogen persists and from which infection is transmitted to a population of concern i.e. a target population (‘Identifying Reservoirs of Infection’, 2002). Domestic dogs are considered maintenance hosts in the reservoir for rabies in many regions, while humans, endangered wildlife, and livestock are often considered target populations (Lembo et al., 2010).
Variant	A viral population maintained within a particular reservoir host in a geographically defined area that

	differs from other viral populations due to either a host shift or diversification within a host species or population i.e. host-association (Lembo et al., 2007) (sometimes called a biotype (Sabeta et al., 2018)). RABV variants often show host-associations. Here we differentiate dog- vs wildlife-associated RABV variants (Fisher et al., 2018).
Directionality of transmission	The predominant direction of transmission from one host species, population or location to another. Genomic data can be used to identify how infected hosts are linked to each other, and to infer the source of infection (Rose et al., 2019). Transmission networks involve mapping transmission routes or pathways to ascertain who infected whom and have been inferred for RABV using parsimony-based approaches and advanced Bayesian frameworks (Rose et al., 2019).
Transboundary spread	Pathogen spread across administrative boundaries. We differentiate human-mediated spread, often over long distances e.g. >50km, from local dispersal due to host behaviour (rabid dogs typically bite animals within 1 km of their location, but can sometimes run over 20 km) (Mancy et al., 2022). An incursion (or introduction) is the spread of a pathogen into a new area, either where that pathogen was historically absent, had been previously eliminated (i.e. re-introduction), or where the pathogen is already present. In the latter example, an incursion might be identified from genetic data when a distinct viral lineage is found in an area where other lineages are circulating (Bourhy et al., 2016; Zinsstag et al., 2017).

Phylogeny or phylogenetic tree	A branching diagram or tree showing the evolutionary relationships between sequences or species. These generally incorporate nucleotide substitution models and can include taxonomic and temporal information. The most widely applied methods for tree building are Neighbour Joining, Maximum Parsimony, Maximum Likelihood and Bayesian Inference.
RABV-Gene-linked by Underlying Evolution (RABV-GLUE)	A flexible software system for interpreting sequencing data with functionality for storage and interpretation. The software is freely available and can be directly downloaded for viral sequence analysis or can be used via the web interface (David Baum, 2008): http://rabv-glue.cvr.gla.ac.uk.
Clade	A monophyletic group with a single common ancestor (David Baum, 2008). Shared ancestry therefore defines the initial pathogen emergence and spread (Pybus & Rambaut, 2009). RABV is designated into clades or subclades (sometimes referred to as major and minor clades), usually associated with specific geographic areas and/or hosts. A lineage (sometimes referred to as a subtype) is a group of related sequences (typically a smaller monophyletic group contained within a larger subclade) defined by statistical support of their phylogenetic placement and genetic differences from their most recent common ancestor (MRCA) (Dellicour et al., 2019). Clades and subclades can be classified differently depending on the hosts and geographical location. In this review, publications may have designated clades/subclades/lineages according to the author's naming system. To allow for comparison between publications, a table of publication-specific

	names and corresponding RABV-GLUE designations is available in A.1 Supplementary Files Table A. 1. Clade designations from various publications and their corresponding RABV-GLUE classifications.
Phylodynamics	The study of how epidemiological, immunological, and evolutionary processes (inter)act to shape viral phylogenies. Examples include studies of spatial diffusion, sometimes incorporating geographical or population structure i.e. phylogeography (Volz et al., 2013).
Genomic/ genetic surveillance	Surveillance involving sequence data to characterise an infectious agent and infer additional information about its dynamics. Genomic refers specifically to Whole Genome Sequencing (WGS) i.e. the entire pathogen genome (>10kb for RABV), whereas partial genome sequencing is the generation of a sequence of a specified genetic region that can be used to identify the organism. For RABV, 400 bp fragments are used for diagnostics, and many phylogenetic studies sequence the N gene, G gene and/or G-L intergenic regions.
1st generation (or Sanger) sequencing	Type of sequencing which produces DNA fragments labelled by chemical modified nucleotides (dideoxynucleotides) during nucleotide elongation. Sanger sequencing sequences one fragment at a time (Kchouk et al., 2017).

Next generation sequencing (NGS)	Parallel approaches that sequence millions of fragments simultaneously, hence have higher throughput than 1st generation approaches. NGS technologies are divided into 2nd generation technologies which analyse clonal representations of the input DNA before sequencing amplified DNA clones, e.g. Illumina MiSeq and Ion Torrent, versus 3rd generation single molecule sequencing technologies, which can produce longer reads than 2nd generation platforms but typically have higher error rates, e.g. Oxford Nanopore and PacBio (Kchouk et al., 2017).

Phylogenetic analysis enables further classification of RABV diversity into clades, subclades and lineages. The RABV genome is 12 kilobases (kb) in length (Pant et al., 2013), comprising five genes encoding the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large polymerase protein (L) (Wunner et al., 1988). Like other RNA viruses, RABV exhibits elevated mutation rates because of the absence of proofreading (Voloch et al., 2014). These mutation rates foster genetic diversity, facilitating tracking of viral spread and enhanced understanding of viral dynamics.

There is no treatment for rabies once clinical signs begin, but post-exposure prophylaxis (PEP) correctly administered shortly after exposure is almost 100% effective in preventing the fatal onset of disease. However, a highly effective canine vaccine is available to prevent disease in the primary reservoir and therefore prevent transmission to humans. Canine rabies elimination is possible through mass dog vaccination, as demonstrated in Europe, North America, and parts of Asia and Latin America. Several countries where dog-mediated rabies was endemic have now been declared rabies-free or are approaching elimination as a result of sustained dog vaccination. According to the World Health Organisation, to eliminate dog-mediated rabies vaccination campaigns need to achieve coverage of at least 70% and be conducted annually for at least three years. Rabies incidence in Latin America has declined dramatically over recent decades due to coordinated regional dog vaccination programs (WHO, 2018). In contrast, most LMICs in Asia and Africa have not allocated sufficient budget to control this disease, and access to dog vaccines remains limited. Compounding the

lack of vaccines, typically, rabies surveillance has also been poor. Additional challenges to rabies control include lack of understanding of dog ownership patterns, population sizes, and accessibility for vaccination as well as cultural practices including dog meat consumption (Nguyen et al., 2024). To address these challenges, international organisations joined forces under the United Against Rabies collaboration to advocate for the global goal of ‘Zero by 30’; to end human deaths from dog-mediated rabies by 2030 (Minghui et al., 2018).

Surveillance plays a critical role in infectious disease control (Cutts et al., 1993). Surveillance entails the continuous, systematic collection, analysis, interpretation, and timely dissemination of health-related information (Thacker & Berkelman, 1988), serving as the foundation for planning, execution and evaluation of public health strategies. For instance, surveillance provides data on the effectiveness of interventions, supporting decision-making for initiatives like 'Zero by 30' (Wallace et al., 2015). Increasingly, surveillance also involves genetic data, for pathogen diagnosis and risk assessment, as well as to identify the source of outbreaks and to characterise pathogen spread (Talbi et al., 2010). Linked with locations, pathogen genetic data have uncovered disease movement; from global migration dynamics to local transmission pathways for pathogens such as Influenza virus (Bedford et al., 2010; Lemey et al., 2014), Ebola virus (Quick et al., 2016), Zika virus (Quick et al., 2017), Yellow fever virus (Faria et al., 2018; Giovanetti et al., 2023), Mpox virus (Forni et al., 2022; Kugelman et al., 2014; Li et al., 2023; Roychoudhury et al., 2023) and SARS-CoV-2 (Faria et al., 2021). Sequencing approaches have the potential to enhance rabies surveillance and provide actionable information to inform control programs locally and globally as part of ‘Zero by 30’. For example, viral sequence data can distinguish undetected local circulation from incursions and potentially identify their sources (Trewby et al., 2017). More generally, sequencing could provide insights into how rabies circulates within populations and the processes responsible for its maintenance in specific localities (Layan et al., 2021; Talbi et al., 2010).

Use of pathogen sequence data for surveillance is, however, not yet routine in most LMICs. Constraints include lack of local sequencing capacity, competent personnel and laboratory resources, and these are affected by costs of, and access to, reagents and consumables, as well as power supplies and cold chain (Quick et al., 2016). Sequencing technologies have become more affordable and efforts are underway to improve accessibility (Brunker et al., 2020). Indeed, growth in sequencing capacity during the COVID-19 pandemic provided evidence of the feasibility of scaling up molecular diagnostics but also highlighted operational challenges. For example, in Nigeria, the number of laboratories capable of

molecular identification of SARS-CoV-2 increased from four to 72 in 2020 (Okeke & Ihekweazu, 2021). In this systematic review, our goal was to examine the extent of the application of genetic approaches to RABV surveillance in regions with endemic dog-mediated rabies (much of Africa, Asia, and parts of Latin America) and how, going forward, these approaches can contribute to the 'Zero by 30' goal.

2.3 Methods

This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021), following a protocol developed a priori to ensure methodological reproducibility and transparency (Supplementary File A.1.1 Viral Sequencing to Inform the Global Elimination of Dog-Mediated Rabies - Protocol).

Eligibility criteria: A set of inclusion and exclusion criteria were established to ensure selection of relevant studies while maintaining clarity, rigor and reproducibility in the systematic review. Studies were included if they focused on canine, human, or terrestrial wildlife rabies (excluding bat rabies), and utilized diagnostic or surveillance methods incorporating genetic sequencing data. Accepted molecular diagnostic methods included PCR-based assays (conventional, RT-PCR, qPCR), whole genome sequencing, or partial gene sequencing for rabies detection and characterization. Studies were excluded if they employ serological, immunohistochemical, or antigen-based methods without sequencing data. Additionally, studies were excluded if they were literature reviews without primary genetic data, did not focus on dog rabies or lacked genomic or sequencing data, were not published in English or were duplicate publications.

Search strategy: A systematic literature search was conducted across PubMed, Web of Science and Google Scholar to identify original studies published between 2000 and 2023 related to rabies genomic surveillance strategies. The search strategy incorporated Boolean operators (AND, OR), truncation (*), and phrase searching ("") to enhance retrieval accuracy and reproducibility. The primary search terms used were: "Rabies AND (genom* OR sequenc* OR molecular OR phylo*) AND (control OR surveillance OR eliminat*)". A detailed breakdown of the search terms and filters applied is provided in Table A. 2 including an example of the Medical Subject Headings (MeSH) terms used in one of the databases, which can be found in Supplementary File A.1.2 . To ensure comprehensive search, additional manual searches were performed, including hand-searching key journals, and

consulting experts (supervisors and mentors) for unpublished or in-progress relevant studies that might not have been captured by the database searches.

Selection of studies: All retrieved studies were imported into Zotero, a reference management software (*Zotero | Your Personal Research Assistant*, 2011) for organization and deduplication. Duplicate records were identified and removed before proceeding with title and abstract screening, which was conducted based on the predefined eligibility criteria. Studies that met the inclusion criteria underwent full-text review to confirm their relevance and methodological quality. The selection process followed the PRISMA guidelines and was documented using a PRISMA flow diagram (Figure **2.1**). This diagram detailed the total number of studies identified, duplicates removed, studies screened and the final number of included and excluded studies.

Screening: The screening process was conducted in two phases to ensure a systematic and unbiased selection of relevant studies. In the first phase, the titles and abstracts of all retrieved articles were independently screened by three reviewers (GJ and MM for studies from Africa and Latin America; CB for studies from Asia) based on the predefined inclusion and exclusion criteria. Studies that were out of scope were excluded. If an article's abstract lacked sufficient detail, the full text was reviewed before deciding on inclusion. Studies marked for potential inclusion by any reviewer proceeded to full-text screening.

In the second phase, the full texts of remaining studies were assessed by GJ and CB using a standardized eligibility format to ensure compliance with inclusion criteria. During this phase, studies were excluded based on the exclusion criteria. Any discrepancies between reviewers were resolved through discussion through video conference sessions, and if needed, a third reviewer (supervisor) was consulted for consensus.

Data extraction: Data extraction was conducted using a structured Excel-based extraction sheet available in a GitHub repository (https://github.com/RAGE-toolkit/RABV_geneticSurv_review/blob/main/Supplementary_material/Supplementary_Table_4.xlsx) to systematically collect key information from the included studies. The extracted fields included study details (authors, year of publication, country, and study aim and study design). To assess the scope of genomic surveillance, data on sample sources (species from which samples were collected and number of samples tested), sample types (brain, saliva or any other tissue) were recorded. Additionally, genomic sequencing information, such as sequence type (WGS or partial genome, with the length and section

sequenced), sequencing platform used, and type of phylogenetic analysis performed, was documented.

To evaluate the impact of genomic data on rabies control, the study outcomes were extracted, including phylogenetic relationships, identified mutations, epidemiological insights, and recommendations for rabies surveillance and control strategies. GJ and CB performed the data extraction to ensure accuracy and consistency, with discrepancies resolved through discussion or consultation with a third reviewer if necessary. This structured approach ensured a comprehensive and reproducible data collection process, facilitating meaningful synthesis and analysis of findings.

Data synthesis: The main characteristics of the studies were summarised (as per the data extraction proforma) using a descriptive narrative approach. To enhance data interpretation and visualization, graphical representations were generated using R (version 4.0.3) (Ihaka & Gentleman, 2000). This included summary statistics to assess study distribution, and geographic mapping of genomic surveillance efforts. Studies were categorized based on methodological approaches, sequencing platforms, and key epidemiological insights, providing a comprehensive overview of current rabies genomic research.

2.4 Results

2.4.1 Study Selection

The database search identified 1558 publications, of which 161 were excluded as duplicates using automation software (Ouzzani et al., 2016). Manual searches identified an additional 23 relevant publications. After screening and assessing eligibility, 220 were retained for systematic review (Figure **2.1**).

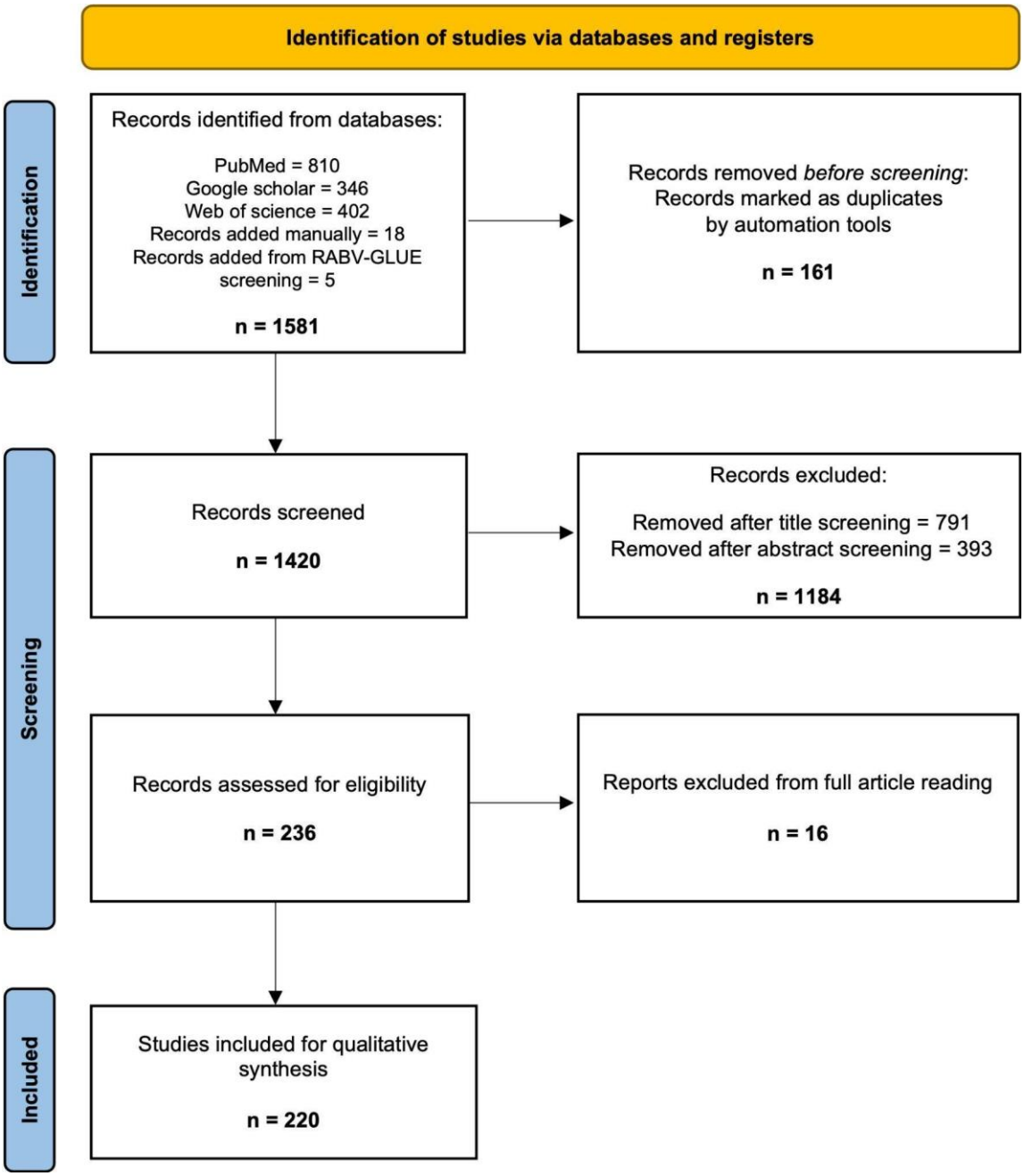

Figure 2.1 **Flow diagram of the article selection process following PRISMA guidelines (*PRISMA 2020 Flow Diagram*, 2020)**

2.4.2 Study Characteristics

The 220 articles generated new RABV sequences from 94 countries (Figure **2.2**), with most from China (n=54 publications) and Brazil (n=19) while six undertook large-scale meta-analyses with additional sequencing from multiple countries (Bourhy et al., 2008; Hayman et al., 2016; Oem et al., 2014; Talbi et al., 2009; Troupin et al., 2016; Velasco-Villa et al., 2017). An average of two studies presenting new RABV sequences were published per year (Figure **2.2**), with most in 2013 (n=16) and 2015 (n=17). All studies generated RABV

sequences from brain tissue samples, with some on FTA cards (Nadin-Davis et al., 2007; Pant et al., 2013; Tenzin et al., 2011; Zeynalova et al., 2015) and four including alternative sample types (nuchal biopsy, cerebrospinal fluid and salivary glands). Most publications (n=188) reported results from partial sequences only, using 1st generation sequencing, mostly the N gene (n=119). Other studies sequenced the G, P or M genes or the G-L intergenic region, and 54 were multi-gene (Figure **2.3**). Twenty-nine studies generated WGS, with hotspots in China (n=10) and Tanzania (n=5), and nine used multiple platforms (1st and 2nd (Campbell et al., 2022; Carnieli et al., 2012; Johnson et al., 2004; Nadin-Davis et al., 2003; Sato et al., 2006; Troupin et al., 2016) or 1st and 3rd generation platforms(Horton et al., 2013; Ismail et al., 2020)). In the last decade, 3rd generation sequencing (Nanopore) increased, as did sequencing output. In most instances, WGS generation was associated with the use of Oxford Nanopore Technologies (ONT), reflecting the growing accessibility of portable, cost-effective sequencing platforms. The increasing preference for WGS is driven by its higher resolution and suitability for standardised phylogenetic analysis (Brunker et al., 2020; Campbell et al., 2022) and enhanced rabies surveillance through integrating WGS with partial genomes resulting in more refined phylogenetic inferences (Holtz et al., 2023).

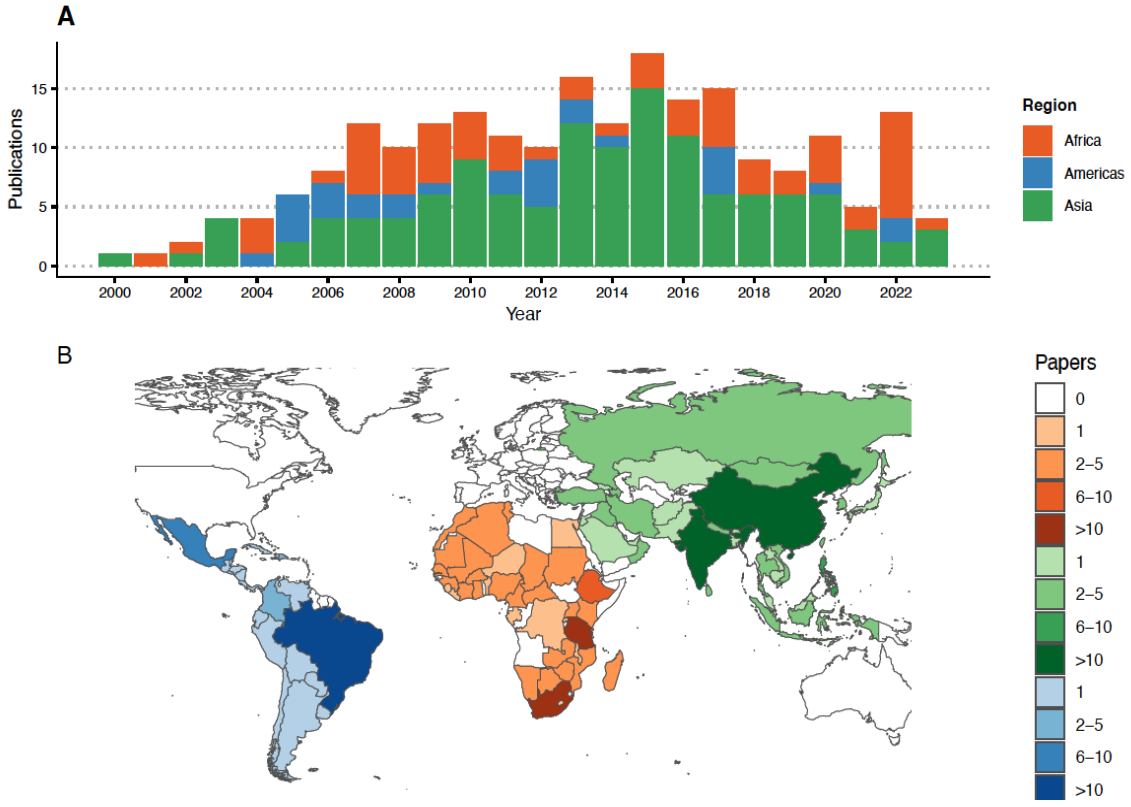

Figure 2.2 **Publications reporting RABV sequences by region from 2000 to 2023.** A) Stacked bar chart showing the number of rabies genomics publications over time, categorized by region (Asia, Americas, and Africa). B) Map of the world showing numbers of publications reporting sequence data by country, shaded by the number of publications and coloured by geographic region. Each publication was attributed to one or multiple countries based on the origin of the RABV sequences, including five studies reporting travel-

associated human cases according to the country of origin. One study describing the global distribution of lyssaviruses **(Townsend et al., 2013a)**, was excluded from this figure.

Sequences were generated from 94 of 149 countries in endemic regions (61.7%). Among the endemic regions, countries in Africa conducted the least in-country sequencing (42.8%, 14/27) whereas countries in Asia conducted the most (76.6%, 23/30, Figure **2.3**). In terms of sequencing output, Asia led with 6,715 sequences (381 WGS), followed by Africa with 3,757 sequences (315 WGS) then Latin America with 1,143 sequences (26 WGS). Tree-building methods used for analyses in publications included Neighbour Joining (n=76), Maximum Likelihood (n=63), Bayesian Inference (n=27) and a combination of these methods (n=48). The main study objectives reported were to identify circulating RABV, describe transmission dynamics, including the species infected and responsible for circulation, and to report viral movement. More generally, studies aimed to derive recommendations to inform control.

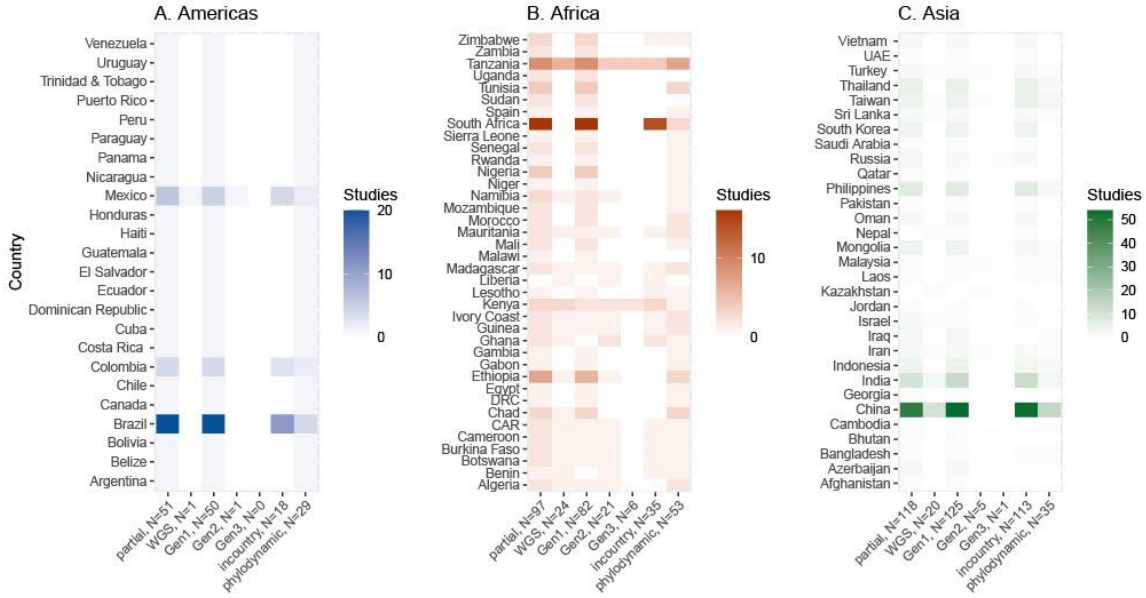

Figure 2.3**. Summary of RABV sequencing by country, including the sequencing platform, type of sequence generated, location of sequencing and analyses undertaken.** The colour intensity increases with the number of publications per country; note the different colour scale by regions; A) Americas, B) Africa, C) Asia. Only twenty-nine publications generated WGS (from 4 countries) and nine conducted phylogeographic analyses. Three publications present data from across multiple countries, with two of these increasing both the number of countries with WGS and phylogeographic analyses**(Talbi et al., 2009; Troupin et al., 2016)**.

2.4.3 Circulating RABV

A large proportion of the 220 studies reviewed described the diversity and distribution of circulating RABVs. Broadly, RABV can be classified into dog-related and bat-related viruses, both of which split into major clades, subclades and lineages (Campbell et al., 2022; Troupin et al., 2016), often associated with geographies and hosts. Publications from Latin America typically differentiated dog-related rabies versus bat-related rabies (Carnieli et al., 2012; Sato et al., 2006; Velasco-Villa et al., 2017), and dog-derived and bat-derived versus skunk variants (Velasco-Villa et al., 2017). In Africa and Asia, RABV diversity was classified predominantly by geographical clustering of dog-related viruses. Generally, there was a lack of standardisation beyond global clade nomenclature, with many studies (n=92) introducing ad hoc names to refer to diversity within global clade assignments. Figure **2.4** uses RABV-GLUE (Campbell et al., 2022) designations to depict circulating clades and a key indicates alternative names used in publications (Table A. 2).

Three RABV clades are reported to circulate in Africa; the 'Cosmopolitan', 'Africa 2' (AF2) and 'Africa 3' (AF3), that subdivide into subclades and lineages (Troupin et al., 2016). The Cosmopolitan clade was found across 27 African countries and split into AF1a, AF1b, AF1c and AF4 subclades. AF1a was broadly distributed across the continent, and predominant in northern and eastern Africa. AF1b was mainly in eastern and southern Africa, while AF1c and AF4 were found in Madagascar and Egypt respectively (Talbi et al., 2009). The AF2 clade is found in 14 countries, mainly across West and Central Africa. The AF3 clade, which is associated with viverrids, is found in Southern Africa, with sequences from South Africa and Botswana (Troupin et al., 2016) (Figure **2.4**). In Asia, RABVs were categorised into four major clades: Cosmopolitan, Arctic (specifically the Arctic-like RABV, which has been found circulating across eastern and southern Asia), Indian Subcontinent, and Asian. The Cosmopolitan clade was widespread in Western (Horton et al., 2013; Ismail et al., 2020; Nadin-Davis et al., 2003; Oude Munnink et al., 2020; Zeynalova et al., 2015), Eastern (Boldbaatar et al., 2010; Feng et al., 2015; Liu et al., 2020; Meng et al., 2011; Tao et al., 2013; Tuvshintulga et al., 2015; Wang et al., 2019; Zhang et al., 2017), and Northern Asia (Deviatkin et al., 2017). Five subclades of the Asian clade (SEA1-SEA5) were found within Eastern and Southeast Asia(Mey et al., 2016; Tohma et al., 2014, 2016; Yamagata et al., 2007). The Indian Subcontinent clade was prevalent in South Asia (Baby et al., 2015; Matsumoto et al., 2013; Nanayakkara et al., 2003; Reddy et al., 2014), with a few sequences from Western and Eastern Asia. While the Arctic-like clade was found in parts of Eastern (Boldbaatar et al., 2010; Hyun et al., 2005; Meng et al., 2011; Shao et al., 2011; Tao et al.,

2015; Yang et al., 2011) and South Asia (Cherian et al., 2015; Jamil et al., 2012; Manjunatha Reddy et al., 2018; Nagaraja et al., 2008; Pant et al., 2013; Reddy et al., 2019, 2014, 2015; Tenzin et al., 2011). In the Americas clades were categorised into dog-maintained and dog-derived variants, including established wildlife foci in skunks, coyotes, grey foxes and mongoose (Velasco-Villa et al., 2017).

Concurrent circulation of divergent clades and subclades within countries, and more locally, was commonly reported across Asia and Africa. This was a less common feature in Latin America, where there are fewer remaining dog-mediated rabies foci and, overall, less dog-related RABV sequence data (almost all partial and just 26 WGS). Six African countries reported co-circulation of the Cosmopolitan and AF2 clades (Cameroon, the Central African Republic, Chad, Ghana, Gabon and Nigeria) (Bourhy et al., 2016; Kia et al., 2018; Sadeuh-Mba et al., 2017; Talbi et al., 2009; Troupin et al., 2016; Zinsstag et al., 2017). One study identified co-circulation of Cosmopolitan AF1b subclade and AF3 clade in Botswana, but from different parts of the country: AF1b in the north and AF3 in the south63. Similarly six African countries reported co-circulation of Cosmopolitan AF1a and AF1b subclades (Cameroon, Chad, Ghana, Mozambique, Kenya and Uganda) (Coetzer et al., 2017a; Gigante et al., 2020a; Hayman et al., 2011; Hirano et al., 2010; Sadeuh-Mba et al., 2017; Zinsstag et al., 2017). All of the four major clades found in Asia were seen co-circulating within and between provinces in China(Feng et al., 2015, 2020, 2022; Guo et al., 2013; Liu et al., 2020; Tao et al., 2013; Tu et al., 2018; Wang et al., 2019). In Southeast Asian countries, only the Asian clade was seen, except in Vietnam where the Cosmopolitan clade was also reported (Yamagata et al., 2007). More differentiated subclades were reported from archipelagic Southeast Asian countries such as Indonesia (Asian SEA1b) (Dibia et al., 2015; Susetya et al., 2008; Susilawathi et al., 2012), the Philippines (SEA4), Sri Lanka (Indian-Sub) (Matsumoto et al., 2013; Nanayakkara et al., 2003), and Taiwan (SEA5) (Chiou et al., 2014; Kj et al., 2016; Yc et al., 2016), with geographically-associated names used. As geographic resolution increased, naming systems became more ad hoc. For example, a study from the Philippines identified nine lineages (GrL1-9) within Group L of SEA4 subclade, named because of circulation within Luzon Island (Saito et al., 2013)

Many studies used nomenclature only meaningful to that study or research group. For example, sequences from China were variously referred to as groups I-IV (Liu et al., 2007; Tao et al., 2009), and lineages A-F (Tao et al., 2013; Zhang et al., 2006). The same nomenclature could represent different diversity across studies. For example, group I referred to SEA1b sequences in one study (Tao et al., 2009), but SEA2a in another (Liu et

al., 2007). Another study defined ‘Indian’ lineages I & II (Reddy et al., 2014), which were re-classified by RABV-GLUE (Arctic NA (i.e. no minor clade assigned), Arctic AL1a, Asian SEA1a, Indian-sub) revealing much broader diversity and geographic distribution than user-defined terminology implied. In Latin America, most studies (n=29) grouped RABVs into lineages named by geographical location or species involved, without following a common nomenclature.

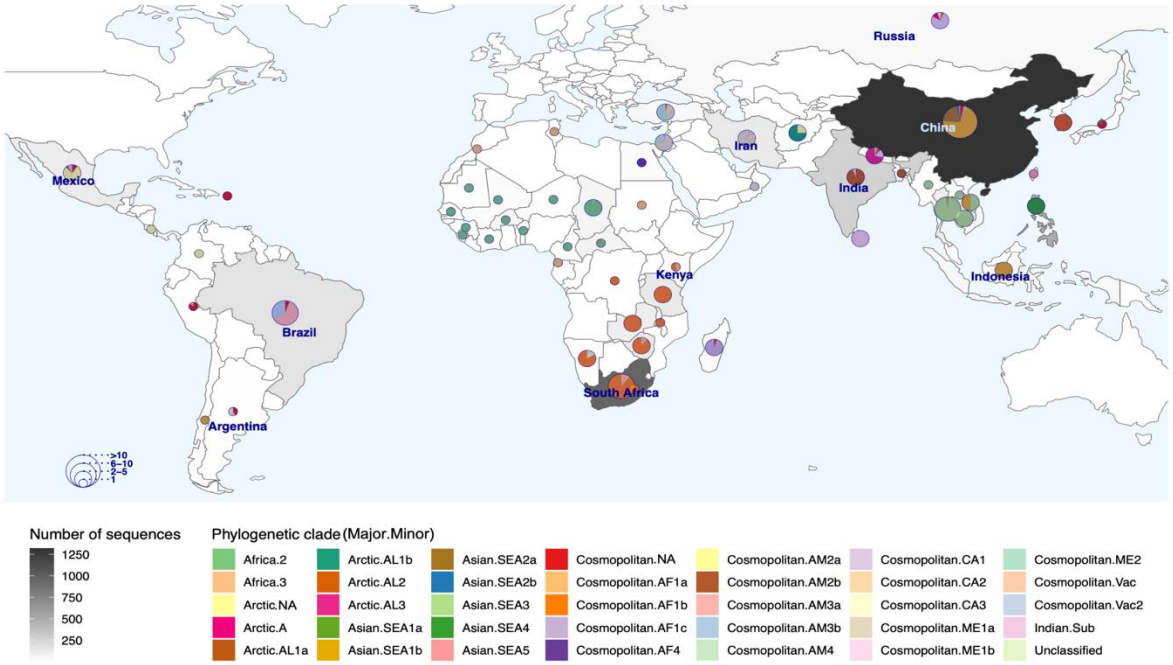

Figure 2.4**. The spatial distribution of dog-associated rabies virus (RABV) clades reported from Asia, Africa and Latin America.** Each country is shaded according to the number of publicly available sequences with at least 90% gene or genome coverage and labelled with a pie chart representing the RABV phylogenetic clades (denoted as Major.Minor clade in the legend e.g. Cosmopolitan.AF1a is major clade Cosmopolitan and minor clade AF1b, while sequences lacking resolution for a minor clade designation are assigned 'NA') within each country and sized according to the number of publications. Note that major and minor clades are annotated according to RABV-GLUE designations(Campbell et al., 2022), but may be annotated differently in primary publications. The correspondence between annotations is detailed in A.1 Supplementary **Files**

Table A. 1.

2.4.4 Host species

Studies reported RABVs from a broad range of domestic and wildlife hosts and humans. Sequences from domestic animals included dogs, cats, cows, goats, sheep, pigs, camels and horses. RABVs were found in wild carnivores such as foxes, jackals, hyenas, mongoose, African wild dogs, wolves, ferret badgers, and civets as well as occasionally in herbivores

(e.g. nilgai and kudu) and more unusual wildlife like monkeys and bears. Eighty-five publications reported sequences from across this species range, 19 from just dogs and livestock, 18 from dogs and wildlife, and 15 from dogs and humans. Cross-species transmission, resulting in spillover, were reported in 63.7% (n=137) of studies: 13.1% (n=18/137) reporting transmission between domestic dogs and wildlife, and 86.9% (n=119) reporting transmission from dogs to both wildlife and livestock. In regions with endemic dog-mediated rabies most livestock outbreaks were attributed to spillover from dogs, except for outbreaks in livestock in Latin America from vampire bat rabies. However, spillover from wildlife to both dogs and to livestock was also observed, as seen in a livestock outbreak linked to foxes in northwest China (Feng et al., 2015). Atypical cases, such as rabies in a chicken bitten by a dog, also highlight spillover events in endemic areas (Baby et al., 2015).

Domestic dogs were predominantly identified as the reservoir of RABVs within the defined scope of this study. Successful control of dog-mediated rabies across Latin America has left bats as the main RABV reservoir, but emergence of independent wildlife-associated cycles were attributed to spillovers from bat, arctic fox and canine rabies (Garcés-Ayala et al., 2017). For example, RABVs were associated with skunks in north-central Mexico, with coyotes in west-central Mexico, grey foxes in Colombia, crab-eating foxes in Brazil and mongoose in Caribbean islands (Velasco-Villa et al., 2017). Two publications from Africa reported other reservoirs: horizontal transmission of a canid variant (AF1b) in kudu in Namibia (Scott et al., 2013), and the distinct AF3 clade circulating in yellow mongoose in Southern Africa (Johnson et al., 2004). Evolutionary rates inferred from AF3 were outliers from dog-related clades, suggesting host-specific adaptation (Troupin et al., 2016). In Asia, a unique ferret badger variant was found in Taiwan, distinct from other Asian subclades (Lan et al., 2017; Zhao et al., 2019).

Studies examined reservoir dynamics, investigating new variants and quantifying transmission within and between species. For example, an Israeli study showed emergence of a dog-related clade that was distinct from fox-associated clades that were predominant before fox-targeted oral rabies vaccination began. The emerging dog-related clade might have indicated a host shift, but sequence similarity to Turkish dog isolates suggested cross-border introduction as the cause of the clade emergence (David et al., 2009). Bayesian phylogenetic inference from WGS of samples from Turkey spanning from 1999 to 2015 was used to refine the date of a host shift that did occur from dogs to foxes to ~1997 (Marston et al., 2017). A European red fox-associated variant reported from hyenas in Tanzania(East et al., 2001), was concluded to stem from laboratory contamination as other studies found that

sequences from both wildlife and dogs all belonged to typical and closely-related dog-mediated variants belonging to clade AF1b (Lembo et al., 2007). Further analysis confirmed the central role of domestic dogs in maintaining circulation of AF1b in this region (Lembo et al., 2008). In the same setting, sequencing an African civet cat case that was presumed to be spillover of dog-mediated rabies, also unexpectedly identified a novel highly divergent lyssavirus (Marston et al., 2012), and no cases of this variant have been found in dogs. The majority of spillover examples identified were from dogs into wildlife, but a limited number of publications reported spillover from wildlife to dogs or other animals, for example, from Coyote into dogs in Mexico (Velasco-Villa et al., 2017).

2.4.5 Human rabies

In total 24 studies (16 from Asia, 6 from Africa and 2 from Latin America) reported sequences from human rabies cases, with a total of 219 human samples sequenced. Most of these studies (n=19) identified human cases to be the result of dog-mediated rabies only. Three studies reported wildlife exposure only including 1 racoon exposure from Nepal (Oude Munnink et al., 2020) and 2 fox exposures, one from India (Madhusudana et al., 2013) and one from Mexico(Velasco-Villa et al., 2008). The reported raccoon exposure in Nepal may be a case of misidentification by the original authors', as raccoons are not native to the country. The biting animal was more likely a red panda, which is indigenous to Nepal. Given their similar head features, dentition, and ringed tails (Simthsonian's National Zoo, 2024), confusion between the two species is possible. A longitudinal study from South Africa reported a majority of human cases from dog-mediated rabies as well as three cases spread from mongoose (AF3) (Weyer et al., 2011). One of the Latin America publications reported a veterinarian who became infected while handling rabid livestock that was determined through genetic analysis to be vampire bat rabies (Brito et al., 2011).

Several of the human cases occurred in rabies-free countries and were resolved through phylogenetic analysis. Eight were traced to immigrants with exposure histories in countries with endemic dog-mediated rabies where PEP was not received before travel to the (rabies-free) country of diagnosis. Analysis of the patient isolates revealed similarity with cases in postulated countries of origin, including a case in the UK imported from Nigeria (Ogo et al., 2011) and a case in France imported from Mali (Contou et al., 2015), two unrelated cases in Qatar both imported from Nepal (Oude Munnink et al., 2020), and cases imported from the Philippines to the UK (Smith et al., 2003), Japan (Nosaki et al., 2021) and Finland (Rimhanen-Finne et al., 2010), as well one that likely originated from insectivorous bats

involving a Mexican immigrant who was bitten by a fox, and who died in California (Velasco-Villa et al., 2008). Similarly a sequence from a patient in France, with recent travel history to Mali but no known exposure, belonged to AF2 subclade that circulates in West Africa (Contou et al., 2015).

Human cases with prolonged incubation periods were also identified (normal incubation periods range from 1 week to 8 months). One human case in rabies-free Australia was traced to exposure before relocation. The 10-year-old of Vietnamese origin had stayed in Hong Kong before immigrating to Australia 5 years prior to symptoms onset. Phylogenetic analysis revealed the isolate's distinctness from Australian Bat Lyssavirus and Vietnamese RABV lineages; instead grouping with a China-associated lineage, suggesting acquisition in Hong Kong and prolonged incubation (Johnson et al., 2008). Two human cases imported to Japan also had incubation periods exceeding 8 months (Nosaki et al., 2021). Most human cases resulted from bites, with two exceptions in Africa where victims were scratched or licked (Coetzee et al., 2008; Weyer & Geertsma, 2011). The first case involved a 26-month-old child scratched by a puppy in Gauteng Province, South Africa. Genetic analysis linked this case to an outbreak that spread in dogs in southern Johannesburg following an introduction from KwaZulu-Natal (Weyer & Geertsma, 2011). In the second case, a 6-year-old child from KwaZulu-Natal had no history of being bitten. However, a neighbour's dog died of unknown causes three days before the child's death. Although rabies was not suspected or tested for in the dog at the time, the authors speculate that this dog was the child's most likely source of exposure to rabies, possibly through non-bite contact with saliva (e.g. lick or scratch). Phylogenetic analysis of other cases circulating in the region identified genetic similarity to the child's sample, further supporting the hypothesis that the child was infected through local exposure (Coetzee et al., 2008).

2.4.6 Rabies virus

Phylogeographic analysis identified local transmission and long-distance movement of rabies viruses based on the geographic association or displacement of clades, subclades and lineages. Some studies revealed situations characterised by sustained local circulation, with distinct lineages and closely related sequences confined within specific geographic areas. For instance, in Nigeria, sequences within AF2 (AF2-1 and AF2-2) clustered in Northern and Southern Nigeria respectively (Ogo et al., 2011). Similar clustering was observed in India (Manjunatha Reddy et al., 2018; Reddy et al., 2014) and major island groups in the Philippines (Mahardika et al., 2014; Tohma et al., 2016). However, local circulation was

often not confined by political borders across contiguous landscapes. For example, samples from the border between Brazil (Mato Grosso do Sul state) and Bolivia clustered, reflecting frequent movement across this boundary (Queiroz et al., 2012).

Most publications mentioned human-mediated long-distance viral movement but only about 25% substantiated their claims through phylogeographic analysis. In Tanzania, discrete phylogeographic analysis revealed long-distance movement between regions (>750 km apart) (Brunker et al., 2015), and sequences from distant ecosystems (Serengeti and Tarangire, >200 km apart) grouped together (Lembo et al., 2007), with seasonal migration of pastoralists (and their dogs) proposed as an explanation. In Asia, long-distance movement was attributed to waves of human migration from China to Southeast Asia (Gong et al., 2010). Indeed, increased trade between and within countries (Tao et al., 2019), including animal trade (Guo et al., 2013; Liu et al., 2020)and the dog meat trade (Mey et al., 2016; Tao et al., 2009; Zhang et al., 2014), were identified as playing a role in RABV incursions and expansion.

Three sources of evidence were used to infer transboundary spread: co-circulation of divergent RABVs, clustering of related sequences from adjacent countries/regions and phylogeographic analyses using location data associated with sequences. Studies reported co-circulating divergent viruses in 31 countries, likely caused by introductions that persisted (Bourhy et al., 2016; Coetzer et al., 2017a; Hayman et al., 2011; Omodo et al., 2020; Sadeuh-Mba et al., 2017; Zinsstag et al., 2017). For example, AF1a, AF1b and AF2 were found co-circulating in southern Cameroon, with the AF1b and AF2 sequences most closely related to sequences from the Central African Republic (Sadeuh-Mba et al., 2017). RABVs circulating in neighbouring countries were often closely related, particularly across shared land borders, like Azerbaijan (Zeynalova et al., 2015) with Georgia, Bangladesh (Jamil et al., 2012) with India, and Tibet (Tao et al., 2019) with Nepal. There were also instances of emerging subclades within one country closely connected to another. For example, Nepalese isolates identified as a new lineage within the Arctic-like (AL-1) subclade that circulates in India (Pant et al., 2013). Indeed, some studies aimed to quantify transmission between countries (Feng et al., 2020; Wang et al., 2019). One study highlighted the importance of China, suggesting the country was a source of translocation to 12 Asian countries due to migration and trade (Meng et al., 2011). Similarly another study used Bayesian inference to test the hypothesis that AF2 was introduced to Ghana from other West African countries with results suggesting spread from Nigeria (Hayman et al., 2011).

Several studies used phylogeographic analyses to infer RABV spatiotemporal dynamics (Gong et al., 2010; Tohma et al., 2014; Wang et al., 2019; Yc et al., 2016; Yu et al., 2012), particularly the direction and speed of dispersal (Carnieli et al., 2013; Dellicour et al., 2019; Meng et al., 2011; Tian et al., 2018). Faster dispersal was associated with anthropogenic factors. Dog-associated lineages in Brazil dispersed at 30.5 kilometres per year (km/yr) in comparison to a lower rate, 9.5 km/yr, in crab-eating foxes, which was attributed to human activities driving dispersal of dog-associated lineages (Carnieli et al., 2013). A high velocity of dog-meditated rabies (18.1 km/yr), was estimated in Iran, using a novel analytical framework, revealing spread linked to accessible areas associated with high human density (Dellicour et al., 2019). This estimate is similar to the average dispersal rate in Northern Africa (19.5 km/year) where, again, landscape accessibility was an important driving factor (Dellicour et al., 2019; Talbi et al., 2010).

The time of introduction and history of rabies spread in various areas was investigated. A publication used historical records and phylogenetic analysis to show that rabies was only present in bats and skunks in the Western Hemisphere, with canine rabies rare or absent among dogs of Native Americans, before the arrival of new dog breeds imported during European colonisation (Velasco-Villa et al., 2017). A more comprehensive recent study combined partial and whole genome sequences to reconstruct movement more precisely, revealing how colonial empires influenced the global spread of rabies viruses (Holtz et al., 2023).

Human-mediated long-distance movement was identified through phylogeographic analysis as the source of incursions into previously rabies-free areas, posing a challenge to maintaining rabies freedom. Examples identified introductions to historically rabies-free areas such as island provinces in Indonesia (Mahardika et al., 2014) and the Philippines(Tohma et al., 2016), and to areas where rabies had been eliminated such as Pemba, (an island off mainland Tanzania (Lushasi et al., 2023)), Gauteng Province in South Africa, and N'Djamena, Chad (Zinsstag et al., 2017).

Geographical features were discussed in fifteen papers, with rivers, lakes and mountain ranges shown to be natural barriers (Coetzer et al., 2017b; Muleya et al., 2019; Talbi et al., 2010). For example, sequences within AF1b subclades (AF1b-1 and AF1b-11) were separated by the Zambezi river and Kariba lake in Zambia and Zimbabwe respectively (Muleya et al., 2019). Transboundary spread between Lesotho and KwaZulu-Natal in South Africa was limited by the Drakensberg and Maloti mountain ranges, which constrain

movement of people and animals (Coetzer et al., 2017b). In Asia, three lineages from SEA5 found in ferret badgers were segregated by mountain ranges and rivers (Yc et al., 2016; Zhao et al., 2019). Transmission corridors that facilitated dissemination included: (1) transportation networks, (2) the presence and size of dog populations (tied to human populations) and (3) anthropogenic factors (trade, agriculture and urbanisation). For example, in Southeast Brazil, urbanisation was reported to play a central role; dog population size was correlated with human populations, meaning higher density regions had more dogs and therefore more RABV diversity (Carnieli et al., 2011). Road networks were often shown to be associated with increased movement, consistent with human-mediated transport of incubating animals. RABV detected from different cities in Mozambique were closely related, despite long distances between them (Coetzer et al., 2017a). Sequences from the AF2 clade were proposed to result from an introduction to Bangui, the capital of the Central African Republic, as the closest related sequences were from neighbouring Chad (Bourhy et al., 2016). In Asia, transport routes were correlated with the RABV distribution in Thailand (Denduangboripant et al., 2005), and China (Ma et al., 2017). At more local scales in Tanzania phylogeographic analysis revealed that presence of dogs, rather than density, predicted spread (Brunker et al., 2018).

2.4.7 Rabies control

All studies provided recommendations for rabies control and prevention, but most were generic and not specifically inferred from sequences. These studies recommended mass vaccination of dogs, as well as oral vaccination in specific wildlife populations where variants had emerged. Other recommendations included dog population management (Deviatkin et al., 2017; J. Ma et al., 2019; Tao et al., 2009; Yuan et al., 2017), monitoring the health of animals for trade and consumption (M. G. de Brito et al., 2011; Kia et al., 2018; Liu et al., 2020; Yamagata et al., 2007), and raising awareness, particularly in communities identified as “high-risk” (Brunker et al., 2018; Ma et al., 2019; Zhang et al., 2013). Only 33 publications provided targeted recommendations grounded in genetic evidence. Some reported spillover events, emphasising the necessity for enhanced surveillance (n=12) for specific wildlife populations (Feng et al., 2015; Nadin-Davis et al., 2007; G. B. M. Reddy et al., 2019; R. V. C. Reddy et al., 2015). Outbreak investigations pinpointed sources of incursions, and recommended monitoring for animal transport and at borders(Mahardika et al., 2014; Matsumoto et al., 2013; Mehta et al., 2016; Tohma et al., 2016) plus surveillance and control measures in rabies-free areas or areas with low-incidence, where introductions pose risks (Lushasi et al., 2023; Tao et al., 2015). Likewise, identification of novel variants

and genetic diversity prompted suggestions for oral vaccines and baits tailored to hosts, e.g. for ferret badgers (Lan et al., 2017; H.-L. Zhang et al., 2014). There were no instances of vaccine-derived cases/outbreaks in the review, but genomic surveillance would be a crucial tool to identify and monitor such occurrences.

Widespread coexistence of diverse lineages and insights into their evolutionary history, transmission dynamics and dispersal rates from more complex phylogeographic analysis, highlighted the urgency of addressing transboundary transmission, which requires coordinated effort(Brunker et al., 2015; Horton et al., 2013; Yamagata et al., 2007). Vaccination campaigns focusing solely on urban localised dog populations have demonstrated short-lived success due to rabies circulation across land borders. Examples include Chad and Central African Republic (Bourhy et al., 2016; Zinsstag et al., 2017), India and Bhutan (Tenzin et al., 2011), and Peru and Brazil bordering Bolivia (Vigilato et al., 2013). The same was true between islands, for example Pemba, off Tanzania (Brunker et al., 2018), and within and between archipelagic countries in Southeast Asia(Mahardika et al., 2014; Susilawathi et al., 2012; Tohma et al., 2016) and also was identified at the borders of states in India (Gibson et al., 2022). Therefore, recommendations included scaling up dog vaccination beyond urban centres to encompass surrounding rural areas, along with coordinating transboundary dog vaccination to minimise spread into cities or between neighbouring countries or administrative units such as states or provinces (Bourhy et al., 2016; Zinsstag et al., 2017).

2.5 Discussion

In this review, we focused on how genetic data informs understanding of rabies dynamics and its control. Findings from 220 studies demonstrate sequencing as a potentially powerful tool for contributing to ‘Zero by 30’. However, information from sequences is often not fully or consistently synthesised into specific, actionable recommendations. When used effectively, sequencing has been instrumental in tracing incursions into rabies-free areas and highlighting extensive transboundary transmission that necessitates coordination of control nationally and regionally (Lushasi et al., 2023; Mahardika et al., 2014; Tohma et al., 2016). Although RABV diversity differs across these regions with endemic dog-mediated rabies, spillovers and transboundary movement was repeatedly reported, emphasising the importance of coordinated transboundary vaccination efforts and surveillance. As countries approach the “endgame”, i.e. the final stages of an elimination programme where disease is still circulating but at much reduced levels, genetic data is expected to become increasingly

useful, providing greater insights for monitoring emerging issues such as spillover and adaptation to alternative hosts and potential re-emergence in dogs.

A major challenge is how classification of phylogenetic diversity and associated nomenclature, beyond the clade level, is not standardised and how varied terminologies (subtypes, subclades, subclasses, clusters etc) are used. In most publications from Africa and Asia, groupings were designated numbers or letters based on subjectively defined clusters in phylogenies, while Latin American studies often employed antigenic variant classification rather than evolutionary (phylogenetic) relationships. Inconsistent terminology hampered a clear understanding of circulating lineages and their geographic distribution, and hindered their use as reference points for further research (Campbell et al., 2022). Many studies used nomenclature only meaningful within the context of that particular study and classifications often differed across related studies (A.1 Supplementary Files

Table A. 1); employing RABV-GLUE (Campbell et al., 2022) to classify minor clades revealed these inconsistencies. The significance of discerning lineages lies in implications for control, for example, differentiating incursions from undetected local transmission and identifying the scale of circulation. Variations in the portion of the genome sequenced (gene-specific) and sequence length contributed to inconsistencies. Most publications used partial genome sequencing, often targeting the N gene, a relatively conserved region used as a diagnostic marker. However, this limited sampling missed important variation, which may define rare and divergent lineages or improve resolution across narrower spatio-temporal windows. Over time, there has been a notable shift toward WGS, particularly in the African region, where recent studies are increasingly adopting WGS to enhance phylogenetic resolution and epidemiological tracing. Despite this progress, WGS still accounts for only 20% of publications, yet it offers deeper insights into viral evolution, transmission dynamics, and cross-species spillover.

We found an urgent need to increase in-country sequencing capacity in endemic countries, with relatively few generating sequences in-country. Regionally, Africa lagged behind Latin America and Asia in terms of capacity, but had a higher output than Latin America, likely because few countries in the region remain endemic for dog-mediated rabies. Most sequencing was driven by research, with routine sequencing not yet part of surveillance in endemic regions. Consequently, there is a shortage of sequences, with largest contributions from China and Brazil (upper-middle-income countries with existing networks and resources), whereas several countries had limited representation: some with just one

publication. The scarcity of sequences, which poses a challenge to characterising RABV diversity and understanding transmission, could be due to different cultural, intrinsic and socio-economic factors. In the aftermath of the COVID-19 pandemic, sequencing capabilities have expanded, becoming more affordable and accessible. This increased capacity has practical applications for responding to rabies outbreaks (Lushasi et al., 2023).

The expansion of genomic sequencing capabilities, accelerated by the COVID-19 pandemic, has greatly enhanced real-time rabies surveillance, enabling faster outbreak detection, tracking of viral spread, and improved implementation of control measures. In addition to supporting immediate outbreak response, genomic data plays a crucial role in longer-term planning, such as identifying and prioritising areas that serve as frequent sources of virus reintroduction. The adoption of portable sequencing platforms has further advanced these efforts by enabling on-site sequencing in resource-limited settings, significantly reducing delays associated with sample transport (Brunker et al., 2020). These innovations have facilitated the near real-time generation of actionable insights for public health decision-making.

For example, in South Africa, genomic sequencing has been used to differentiate wildlife reservoirs from dog-mediated transmission, allowing for more targeted control strategies (Coertse et al., 2017). In the Philippines, integration of genomic surveillance with traditional epidemiological methods helped uncover an outbreak in a previously to inform targeted rabies-free area, revealing at least three independent human-mediated virus introductions from neighbouring endemic provinces (Chapter 5). These case studies illustrate the increasing utility of near real-time sequencing in informing both short-term responses and long-term rabies control strategies, underscoring its essential role in achieving the global "Zero by 30" target.

Although dogs were identified as the primary host responsible for most transmission in regions with endemic dog-mediated rabies which were the focus of this study, RABVs broad host range means it is capable of transmission among multiple species. This versatility creates potential for new reservoirs, raising concerns about the effectiveness of control measures, and about these reservoirs being a source of re-emergence (S. Zhang et al., 2013). In areas where dog-mediated rabies has largely been eliminated, there were several examples of transmission cycles in wildlife, some of which resulted from spillover from dogs. In Latin America, where canine rabies incidence has reduced dramatically from coordinated regional control, intriguing reservoir dynamics have emerged. For example, three major enzootic

cycles, distinct from dog rabies foci in Mexico (skunks, coyotes and gray foxes) highlight a complex maintenance dynamic (Velasco-Villa et al., 2005). Similarly, transmission cycles have established in ferret badgers independently in both Taiwan and China (Chang et al., 2015; Lan et al., 2017; Zhao et al., 2019). These findings underscore the complex reservoir dynamics of RABV and importance of understanding host associations and cross-species transmission. As dog-mediated rabies declines, this understanding will be necessary to enable targeted control, either directing efforts at blocking transmission and spread between source and target populations or controlling infection within new reservoirs. The risk of rabies spillover from wildlife foci to dogs is a serious challenge for 'Zero by 30'. When such reservoirs exist, tailored surveillance and control measures are crucial to mitigate the threat of re-emergence in dog populations as a result of spillover. This may eventually require countries to undertake control measures in wildlife reservoirs, but more imminently underscores the need to eliminate rabies from dog populations before wildlife foci can establish.

Underreporting and misdiagnosis of human rabies remains a significant issue and can create a false impression of low burden in rabies endemic settings across Africa and Asia. While enhancing diagnostic capacity and overall surveillance is crucial to address underreporting, we also emphasise the pivotal role that genetic data can play in strengthening human rabies surveillance. Rabies-free countries experience imported human cases from exposures in endemic countries (European Centre for Disease Prevention and Control, 2021; Schmiedel et al., 2007; Solomon et al., 2005). Oftentimes, these imported cases were confirmed through epidemiological investigation, but genetic data identified the sources of infections, as well as ambiguous cases, without a clear route of exposure (e.g. no bite history) or origin (e.g. the migrant from Mexico and the veterinarian in Brazil), or with unusually long incubation periods (Coetzee et al., 2008; Johnson et al., 2008; Nosaki et al., 2021; Weyer & Geertsma, 2011). These human cases identified from countries that are free from dog-mediated rabies highlight how these technologies could be applied to strengthen human rabies diagnosis and source attribution within endemic countries.

Unlike infections in humans, which are effectively dead-end hosts, movement of infected animals is the predominant factor contributing to RABV establishment in new geographic settings. Publications highlighted both local host movement and long-distance human-mediated movements, however, evidence on transmission links and directionality were sparse, with only a few studies (25%) employing phylogeographic analyses. Most used genetic relatedness of viruses from different locations, based on interpreting phylogenies,

without quantitative inference. When carried out, phylogeographic analyses illuminated environmental features as both natural barriers and drivers of spread, with human-related factors driving dispersal towards more populated and accessible areas. These analyses have potential to guide spatial targeting of vaccination, and enhancement of surveillance in at-risk areas (Dellicour et al., 2019; Lumlertdacha et al., 2006; Tao et al., 2015). Frequent reintroductions highlighted epidemiological connectivity of landscapes over which vaccination needs scaling and coordination across political/ administrative boundaries, (Zinsstag et al., 2017) with more recent studies showing how sequencing can be used to monitor the impacts of and threats to dog vaccination programmes(Gigante et al., 2020a; Lushasi et al., 2023; Zinsstag et al., 2017).

2.5.1 Broader context

Our review has relevance to the broader application of genetic surveillance to pathogens. The recent increase in third-generation sequencing of RABV has potential to further expand given the focus on sequencing capacity for pandemic response. Deployable sequencing has become a key component of outbreak response, with portable lab equipment and sequencing platforms facilitating on-site, real-time, genomic surveillance

. Feasibility and utility of deployable sequencing for rabies surveillance has been demonstrated (Brunker et al., 2020; Gigante et al., 2020a; Lushasi et al., 2023), with use of Nanopore's MinIon reducing costs and turnaround times (Brunker et al., 2020; Lemey et al., 2014), and comprehensive protocols, bioinformatic pipelines and open-source user-friendly software and classification tools becoming more available (Bautista et al., 2023; Hadfield et al., 2018; O'Toole et al., 2021; Theys et al., 2019). Sequencing for routine surveillance of endemic zoonoses could build and sustain pandemic preparedness at the human-animal-environment interface. While COVID-19 accelerated application of genomic surveillance, it also highlighted stark global disparities in access to sequencing, and bioinformatic expertise remains a bottleneck (A. F. Brito et al., 2022; Ling-Hu et al., 2022) Since the pandemic investment in LMICs has begun but much more is required (Inzaule et al., 2021).

RABV also serves as a model system to understand and manage cross-species transmission and spillover. Instances highlighted in this review, reflect a broader ecological pattern with significant public health and ecological implications. Spillovers can be precursors to larger outbreaks, as evidenced by recent epidemics of Influenza, Ebola, Zika, and COVID-19, while swift sequencing and analysis can inform public health responses and containment

(Bedford et al., 2010; Faria et al., 2021; Kugelman et al., 2014; Lemey et al., 2014; Quick et al., 2016).

2.5.2 Limitations of the study

While we endeavoured to comprehensively review global regions with endemic canine rabies, it is important to acknowledge the limitations inherent in our study. Genetic studies on rabies demonstrating progress in its elimination, conducted in countries or regions such as Canada, the USA, and Eastern Europe, where dog rabies has been eliminated for some time, were not included. This approach may have limited our ability to discover additional interventions and significant insights from the perspective of these countries. Our review, while focusing on publications from endemic regions may have failed to identify publications documenting importations in rabies-free countries. We supplemented our searches by manually adding relevant instances (~10% of papers), but some studies, particularly those published in non-English journals, may have been overlooked. Additionally, manual searches, and expert citation, relied on subjective judgment and were not as systematically reproducible as database searches. Selection bias may also arise if studies were retrieved based on accessibility rather than predefined criteria. Despite efforts to document these searches systematically, the inherent lack of standardization in manual searches may have introduced variability in study inclusion. Despite substantial manual curation efforts in RABV-GLUE to enhance GenBank metadata (Campbell, 2021/2022), inconsistencies and gaps persist, potentially hindering data mining efficiency for sequences not associated with a publication. A manual search was deemed necessary because several known important studies were not retrieved through the systematic searches, as their titles did not explicitly contain the predefined search terms. We therefore retrospectively and manually identified some studies that did not report the use of sequencing in their abstract. Not all papers transparently report methods, in particular many did not report locations where sequencing was undertaken which we assumed was done in-country. We therefore may have overestimated sequencing capacity for some countries, though we expect our conclusions are robust. We included only studies published after the year 2000, which limited the scope of the study, as it did not capture some of the earliest genomic studies conducted on RABV. However, this also meant most of the methods reported were more comparable and aligned with the 1st, 2nd and 3rd generation sequencing platforms defined in Box 1.

2.5.3 Conclusions and future recommendations

The systematic review provided a foundational framework for the rest of the thesis by synthesizing global evidence on rabies genomic surveillance and identifying gaps in sequencing efforts. The findings directed the improvement of a fully operational genomic sequencing workflow tailored for LMICs, ensuring that sequencing approaches were feasible, cost-effective, and optimized for resource-limited settings. Additionally, insights from the review guided the characterization of rabies virus in the Philippines, demonstrating how genomic sequencing can reveal region-, island-, and province-specific viral lineages, facilitating more targeted and locally adapted control measures.

Despite the promise of genetic data for informing rabies control, its full potential remains untapped as most publications advocate generic measures that lack specificity derived from sequencing. More demanding phylodynamic analysis, which integrate geographical, epidemiological, and genetic data to yield more detailed and quantitative insights, require greater expertise and computational resources which remain inaccessible in many LMICs. Future research would benefit from scaling up WGS as well as leveraging the power of existing data through analyses that integrate partial and WGS (Holtz et al., 2023). The applied insights gained by enhancing rabies surveillance with sequencing lie in understanding what is circulating and how it is spreading, allowing for more effective elimination strategies. Establishing a standardised nomenclature system for categorising RABV diversity, would facilitate clear communication and collaboration among researchers, healthcare professionals and policymakers (Campbell et al., 2022).

To achieve this, we recommend the development of a robust taxonomic classification system under the International Committee on Taxonomy of Viruses (ICTV) that can integrate all existing and newly identified RABV sequences. Scaling up sequencing in endemic countries, strengthening laboratory networks, and adopting a more unified terminology for exchanging information on emerging variants and lineages would enhance risk assessment and control strategies. Genetic data further emphasize the need for international and regional coordination in controlling transboundary transmission, to accelerating progress and sustaining elimination efforts. Expanding sequencing initiatives and fostering collaborative efforts will be critical in achieving the ‘Zero by 30’ goal, and will serve as a prime example of a genomics-informed One Health approach, building capacity for the future (Hayman et al., 2023).

Chapter 3

Whole Genome Sequencing for Rapid Characterization of Rabies Virus Using Nanopore Technology

3.1 Abstract

Genomic data can be used to track the transmission and geographic spread of infectious diseases. However, the sequencing capacity required for genomic surveillance remains limited in many low- and middle-income countries (LMICs,) where dog-mediated rabies and/or rabies transmitted by wildlife such as vampire bats pose major public health and economic concerns. We present a rapid and affordable sample-to-sequence-to-interpretation workflow using nanopore technology. Protocols for sample collection and diagnosis of rabies are briefly described, followed by details of the optimized whole genome sequencing workflow, including primer design and optimization for multiplex PCR, a modified low-cost sequencing library preparation, sequencing with live and offline base calling, genetic lineage designation, and phylogenetic analysis. Implementation of the workflow is demonstrated, and critical steps are highlighted for the local deployment, such as pipeline validation, primer optimization, inclusion of negative controls, and use of publicly available data and genomic tools (GLUE, MADDOG) for classification and placement within regional and global phylogenies. The turnaround time for the workflow is 2-3 days, and the cost ranges from $25 per sample for 96 samples run to $80 per sample for 12 samples run. We conclude that setting up RABV genomic surveillance in LMICs is feasible and can support progress towards the global goal of zero dog-mediated human rabies deaths by 2030, as well as enhanced monitoring of wildlife rabies spread. Moreover, the platform can be adapted for other pathogens, helping to build versatile genomic capacity that contributes to epidemic and pandemic preparedness.

3.2 Introduction

The rabies virus is a lyssavirus in the Rhabdoviridae family that causes a fatal neurological disease in mammals (Rupprecht, 1996). Although rabies is 100% preventable by vaccination, it remains a major public health and economic concern in endemic countries. Of the 60,000 human rabies deaths estimated to occur each year, over 95% are in Africa and Asia where dogs are the primary reservoir (WHO, 2021b). In contrast, dog vaccination has led to the elimination of dog-mediated rabies across Western Europe, North America, and much of Latin America. In these regions, reservoirs of rabies are now restricted to wildlife such as bats, raccoons, skunks, and wild canids (WHO, 2018). Across Latin America, the common vampire bat is a problematic source of rabies due to regular spillover transmission

from bats to both humans and livestock during nightly blood feeding (Benavides et al., 2020). The annual global economic impact of rabies is estimated to be 8.6 billion USD, with livestock losses accounting for 6% (Hampson et al., 2015).

Sequence data from viral pathogens combined with metadata on the timing and source of infections can provide robust epidemiological insights (WHO, 2022). For rabies virus (RABV), sequencing has been used to investigate the origin of outbreaks (Chiou et al., 2014; Tsai et al., 2016) identify host associations with wildlife or domestic dogs (Lembo et al., 2008; Sabeta et al., 2007; Scott et al., 2013), and trace sources of human cases (Coetzee & Nel, 2007; Oude Munnink et al., 2020; Smith et al., 2003). Outbreak investigations using phylogenetic analysis have indicated that rabies emerged in the formerly rabies-free province of Bali, Indonesia through a single introduction from the nearby endemic areas of Kalimantan or Sulawesi (Mahardika et al., 2014), while in the Philippines, an outbreak on Tablas island, Romblon Province was proven to be introduced from the main island of Luzon (Tohma et al., 2016). Viral genomic data have also been used to better understand pathogen transmission dynamics required for targeting control measures geographically. For example, genomic characterization of RABV illustrates geographic clustering of clades (Biek & Real, 2010; Saito et al., 2013; Tohma et al., 2014), co-circulation of lineages (Benjathummarak et al., 2016; David et al., 2009; Reddy et al., 2011), human-mediated viral movement (Denduangboripant et al., 2005; Talbi et al., 2010; Tohma et al., 2014), and metapopulation dynamics (Bourhy et al., 2016; Zinsstag et al., 2017).

Genomic surveillance plays a vital role in disease monitoring, which has significantly advanced with the global increase in sequencing capacity during the SARS-CoV-2 pandemic. This approach enabled real-time tracking of SARS-COV-2 variants of concern (Mannsverk et al., 2022; Yakovleva et al., 2022) and guided effective countermeasures (WHO, 2022). Advances in accessible sequencing technology, such as nanopore technology, have led to improved and more affordable protocols for rapid sequencing of both human (Cabibbe et al., 2020; Soufi et al., 2022; Stubbs et al., 2020; Xu et al., 2021) and animal pathogens (Croville et al., 2018; O'Donnell et al., 2019; Theuns et al., 2018). However, a potential challenge in viral genomic surveillance is the occurrence of primer mismatches, which can reduce the efficiency and accuracy of PCR amplification, leading to incomplete sequencing coverage. To address this, optimising primer design and evaluating primers, especially when handling samples from diverse sources, is essential.

In rabies-endemic countries, the implementation genomic surveillance remains limited due to challenges in laboratory infrastructure, supply chains, and technical expertise, as shown by global disparities in SARS-CoV-2 sequencing capacity (Brito et al., 2022). In this paper, we demonstrate how an optimized rapid, and affordable whole genome sequencing workflow can be deployed for RABV surveillance in resource-limited settings, including the importance of primer design.

3.3 Methods

3.3.1 Protocol Adaptation and Workflow Integration

This study utilized a publicly available, optimized whole-genome sequencing (WGS) protocol for rabies virus in LMIC settings, as described by Brunker et al. (Brunker et al., 2020). The protocol was adapted into a comprehensive end-to-end workflow, encompassing sample collection, sequencing and data interpretation (Figure **3.1**). Each step, including sample preparation, multiplex PCR, library preparation, sequencing and bioinformatics analysis, was systematically outlined and integrated into the workflow (B.1 Supplementary Files

B.1.1). The workflow applied a half-volume reaction setup of the original multiplex PCR assay, using 50% of the recommended reagent volumes per reaction. This modification was implemented to significantly reduce reagent consumption and overall assay cost without compromising the assay's performance. Bioinformatics pipeline customised for the protocol was made user-friendly and computationally feasible both for new users and expert users. To enhance accessibility and support users in navigating complex steps of the protocol, a video presentation was created and published in JoVE (Journal of Visualized Experiments). This instructional video provides a visual demonstration of some parts of the protocol, making it easier to follow than text alone. By complementing the written instructions, the video ensures a clearer understanding of challenging steps, allowing users to replicate the workflow more effectively. Additionally, a lineage assignment tool, MADDOG (Campbell, 2021/2021) was used to identify and designate lineages of the newly generated sequences. This approach ensures a standardized and practical method for rabies WGS, facilitating its application in resource-limited settings.

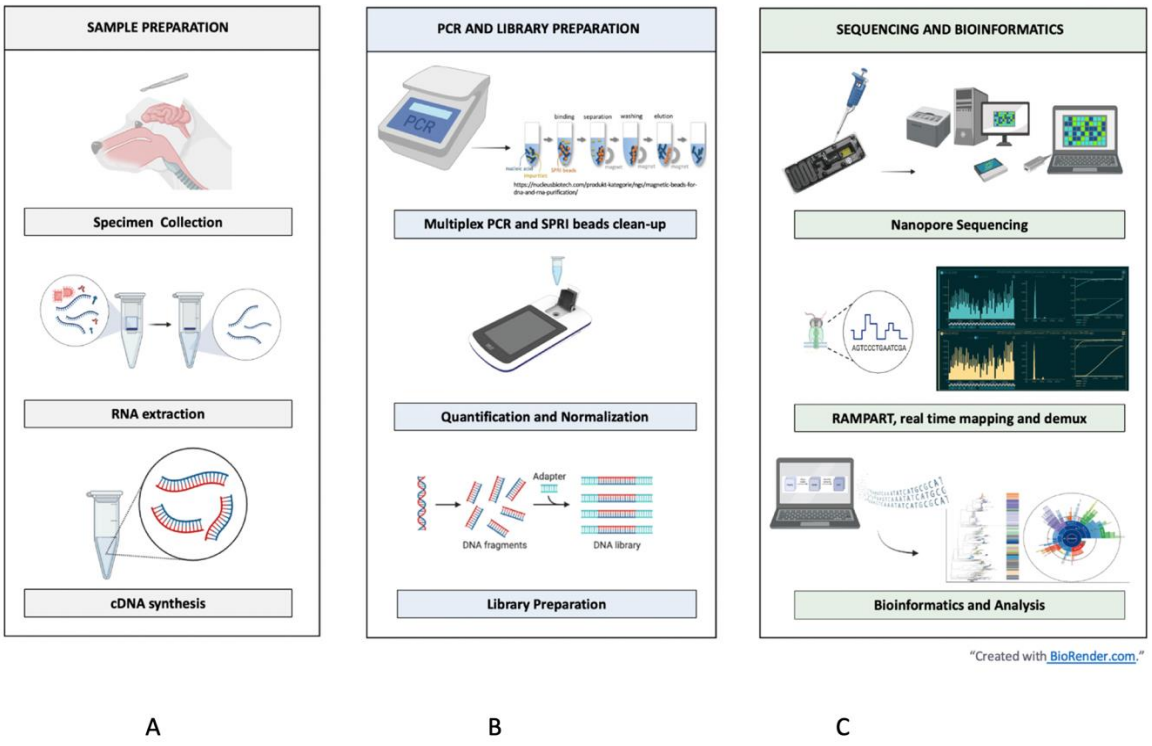

Figure 3.1**. Sample-to-sequence-to-interpretation workflow for Whole Genome Sequencing of RABV.** Summarized steps are shown for (A) sample preparation, (B) PCR and library preparation and (C) sequencing and bioinformatics up to analysis and interpretation.

3.3.2 Benchmark Testing in Different Settings

To evaluate the performance and adaptability of the workflow, benchmark testing was conducted across multiple laboratory settings, ranging from well-equipped reference laboratories and resource-limited facilities. The protocol was applied by several students from Tanzania, Nigeria, Philippines, Peru, as well as researchers from Philippines as part of their sequencing research work. The protocol was tested using samples collected and processed in their respective country laboratory settings. To ensure only rabies-positive samples were included for sequencing, brain tissue samples were pre-screened using Direct Fluorescent Antibody Test (DFAT). The workflow was assessed across various sample types and conditions; including fresh and frozen brain tissue, cDNA and RNA extracts from rabies positive brain samples transported under cold chain for extended periods, and FTA cards containing brain tissue smears from animal samples. Human samples include saliva and skin biopsy sample. All sample types except the RNA extracts were processed for RNA extraction following the prescribed protocol from the commercially available extraction kit used. The kit protocol for RNA clean-up was used on the RNA extracts. The sequencing activities were supported through a funded project, which provided essential reagents,

consumables, and start-up kits sufficient for sequencing a number of samples. This support enabled them to independently implement their research while also evaluating the protocol's feasibility, reproducibility, and applicability within their local laboratory environments.

3.3.3 Cost Assessment of Whole-Genome Sequencing in LMICs

A cost assessment was conducted to evaluate the financial feasibility of implementing the WGS workflow in LMIC settings. The total cost per sample was estimated by analysing key expense components, including sample processing, reagents, sequencing consumables, equipment usage, and bioinformatics resources. Additional factors such as supply chain constraints, maintenance costs, and personnel training were considered to assess long-term sustainability.

3.4 Results

3.4.1 Protocol Adaptation and Workflow Integration

The optimised sample-to-sequence-to-interpretation workflow for RABV was successfully adapted across multiple laboratory settings in endemic countries, including Tanzania, Kenya, Nigeria, and Philippines (Figure 3.2. **Global Distribution of RABV Sequencing Using the Optimized Workflow (2021-2022)). The protocol was applied to a variety of sample types and conditions (**

Live basecalling using RAMPART (Figure **3.3**) facilitated live monitoring of sequencing runs, allowing informed decisions on run duration and flow cell reuse. Run times varied significantly, with some runs completing in two hours, while others required over 12 hours to reach the target depth of coverage (≥ x100).

Figure 3.3. **Screenshot of RAMPART visualization in web browser.** Barcode names are replaced by sample names according to bioinformatic setup. Top three panels show summary plots for the whole run: depth of coverage of mapped reads for each barcode per nucleotide position on the index reference genome (top left, coloured by barcode) summed mapped reads from all barcodes over time (top middle), mapped

reads per barcode (top right, coloured by barcode). Lower panels show rows of plots per barcodes. From left to right: the depth of coverage of mapped reads per nucleotide position on the index reference genome (left), length distribution of mapped reads (middle), proportion of nucleotide positions on the index reference genome which have obtained 10x, 100x and 1000x coverage of mapped reads over time (right).

Sequencing reads were mapped to the reference genome to generate depth of coverage profiles showing the number of reads aligned at each genomic position as shown in an example from the Philippines (Figure **3.4**). This example revealed regions with poor amplification where certain amplicons exhibited very low coverage, suggesting potential primer performance issues. By investigating these poorly amplifying regions more thoroughly, primer mismatches were revealed allowing for the redesign and improvement of individual primers. The East Africa primer scheme exhibited more mismatches compared to the Philippines scheme as it was designed to capture the broader diversity of circulating RABV minor clades (AF1a and AF1b), versus the region-specific SEA4 clade in the Philippines.

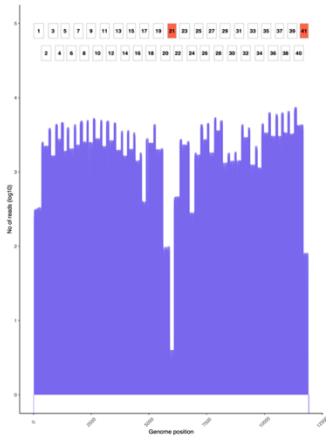

Figure 3.4. **Example sequencing depth of coverage profile for a sample from the Philippines.** Depth of coverage by mapped reads (y axis, $\log_{10}$ scale) is plotted per nucleotide position on the index reference genome (x axis). The approximate position of amplicons (1-41) is labelled at the top of the plot. Spikes in the depth of coverage between amplicons indicate areas of amplicon overlap. Amplicons with a low depth of coverage (such as 21 and 41, with values lower than 2) indicate problematic regions of the genome where primers may require optimization. The $\log_{10}$ scale allows visual assessment of coverage thresholds, where a value ≥ 2 ($\log_{10}$ (100)) represents coverage of at least 100 mapped reads, the minimum threshold for sufficient coverage in downstream analyses.

3.4.2 Benchmark Testing in Different Settings

Whole-genome sequencing using the optimised workflow was benchmarked across various laboratory environments (Table 3.1). In total, 450 samples from eight sequencing runs were

processed across five countries between 2021 and 2022. Performance was evaluated based on sequencing success rates, genome coverage, and lineage assignment accuracy.

Optimisation of primer sets based on coverage analysis helped resolve amplification issues, contributing to more uniform genome coverage. Resulting sequences were analysed using RABV-GLUE and MADDOG tools for major and minor clades and lineage classification (Table 3.2) with higher-resolution lineage assignments revealing local viral diversity. In East Africa, a broad array of Cosmopolitan clade lineages was detected, including sub-lineages from AF1a and AF1b. In contrast, the Philippines exhibited a more homogenous population of Asian SEA4 clade. Nigerian sequences belonged to Africa 2 within the Africa clade.

These results demonstrate the protocol's versatility across different geographic and laboratory settings, enabling high-resolution genomic analysis even in regions with substantial viral diversity.

3.4.3 Cost Assessment of Whole-Genome Sequencing in LMICs

The adjusted multiplex PCR setup effectively reduced reagent consumption and assay costs without compromising performance. The cost assessment revealed the total expense per sample for implementing the WGS workflow in LMIC settings varied significantly depending on regional factors such as reagent availability, supply chain stability, and laboratory infrastructure. The initial computation of sequencing costs was based on reagent and material prices in the United Kingdom, where funding for the pilot sequencing in LMICs was sourced through a collaborative project with the University of Glasgow. The cost per sample ranged from $25 for a 96-sample run to $80 for a 12-sample run.

To assess the feasibility of conducting in-country genomic sequencing, we obtained price quotes from local suppliers in Nigeria and the Philippines. Local pricing revealed increased costs per sample—by 24% in the Philippines (from $80 to $99) and 28% in Nigeria (from $80 to $102). This rise is likely due to higher mark-ups on Nanopore sequencing reagents used in library preparation, which are more expensive than standard PCR reagents and consumables. The elevated costs in these settings are likely driven by importation fees and

persistent challenges in supply chain access. —primarily due to import duties, limited local access to reagents and consumables, and supply chain challenges.

Despite this variability, the workflow remains a viable and cost-effective tool for in-country genomic surveillance programs in LMICs. However, the findings emphasize the importance of strengthening local reagent supply chains and laboratory infrastructure to improve cost-efficiency and long-term sustainability.

Table 3.1. Number of Samples Processed in Different Countries.Table 3.1), including fresh and frozen brain tissue, cDNA and RNA extracts transported under cold conditions and brain tissue smears preserved on for FTA cards. All sample types provided sufficient genome coverage for consensus sequence assembly, with the exception of a human saliva sample collected in the Philippines. A skin biopsy from the same patient yielded improved coverage, highlighting its utility as an alternative sample type.

RNA extracts transported under cold chain conditions also performed well, although minor degradation was noted during RNA quantification. In the Philippines, despite adherence to unidirectional workflow practices, contamination issues were still encountered, primarily traced to reagent contamination. Most library preparations met required DNA input thresholds, although low-yield libraries were occasionally observed and likely linked to insufficient DNA concentrations.

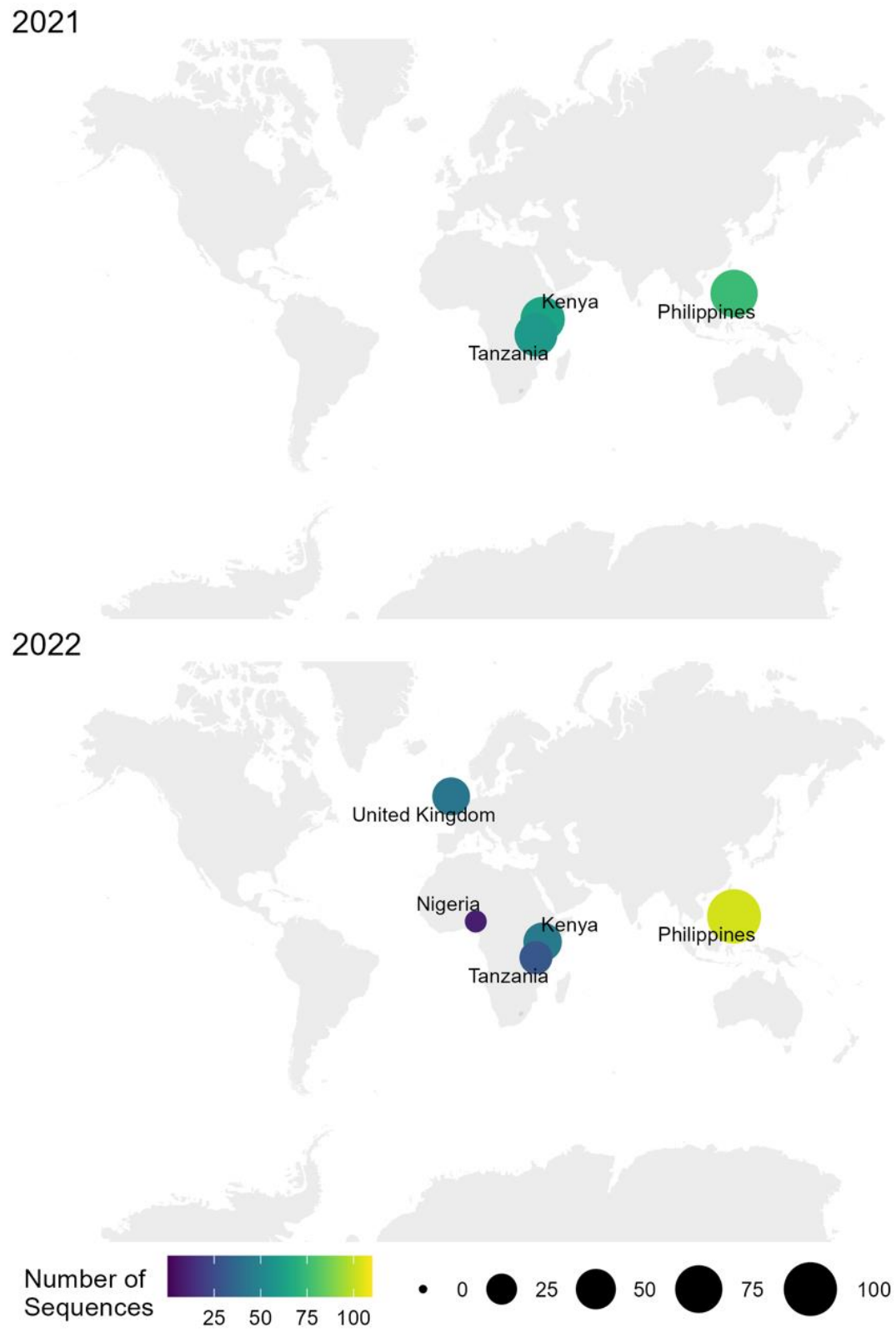

Figure 3.2**. Global Distribution of RABV Sequencing Using the Optimized Workflow (2021-2022).** Map showing the locations where RABV sequencing was conducted using the optimized workflow in 2021 and 2022. The size and color intensity of the bubbles represent the number of sequences generated per location, with smaller/darker bubbles indicating fewer sequences and larger/ lighter bubbles indicating more sequences.

Live basecalling using RAMPART (Figure **3.3**) facilitated live monitoring of sequencing runs, allowing informed decisions on run duration and flow cell reuse. Run times varied significantly, with some runs completing in two hours, while others required over 12 hours to reach the target depth of coverage (≥ x100).

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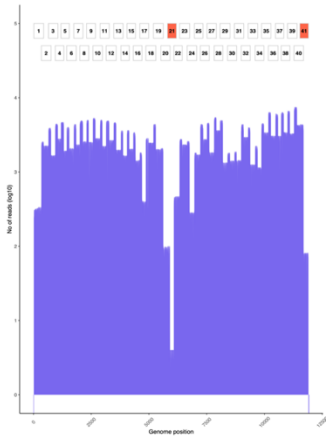

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These results demonstrate the protocol's versatility across different geographic and laboratory settings, enabling high-resolution genomic analysis even in regions with substantial viral diversity.

3.4.5 Cost Assessment of Whole-Genome Sequencing in LMICs

The adjusted multiplex PCR setup effectively reduced reagent consumption and assay costs without compromising performance. The cost assessment revealed the total expense per sample for implementing the WGS workflow in LMIC settings varied significantly depending on regional factors such as reagent availability, supply chain stability, and laboratory infrastructure. The initial computation of sequencing costs was based on reagent and material prices in the United Kingdom, where funding for the pilot sequencing in LMICs was sourced through a collaborative project with the University of Glasgow. The cost per sample ranged from $25 for a 96-sample run to $80 for a 12-sample run.

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Despite this variability, the workflow remains a viable and cost-effective tool for in-country genomic surveillance programs in LMICs. However, the findings emphasize the importance of strengthening local reagent supply chains and laboratory infrastructure to improve cost-efficiency and long-term sustainability.

Table 3.1. Number of Samples Processed in Different Countries. Number of rabies virus whole-genome sequences generated, and sample types used in across various countries implementing the sample-to-sequence-to-interpretation workflow.

Year	Country (City/Province)	# Samples sequenced	Type of samples
2021	Tanzania (Kilimanjaro region, Moshi District)	35	Brain tissue
2021	Kenya (Nairobi)	55	Brain tissue
2021	Philippines (Manila)	75	Brain tissue
2022	UK (Glasgow)	30*	cDNA from brain sample, RNA extracts from brain sample,

			brain tissue preserved on FTA cards
2022	Nigeria (Zaria)	9	Brain tissue
2022	Philippines (Manila, Davao)	134	Brain tissue, human saliva sample, skin biopsy
2022	Tanzania (Kilimanjaro region, Moshi District)	67	Brain tissue
2022	Kenya (Nairobi)	45	Brain tissue

*Country of origin: Nigeria

Table 3.2. Major and minor clade assignments from RABV-GLUE and lineage assignments from MADDOG for sequences generated using the workflow

Region (Country)	Major Clade from RABV-GLUE	Minor Clade from RABV-GLUE	Lineage Assignment from MADDOG
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_A1.2
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_A1.2.1
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_A1.2.2
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_A1.2.3
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_C1.1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_G1
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_J1
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_J1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	NA	Cosmopolitan_K1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1a	Cosmopolitan AF1a_A1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1a	Cosmopolitan AF1a_A1.1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_A1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_A1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_A1.1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_A1.1.2
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_A1.1.3
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_B1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_B1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_B1.1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_B1.2
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_B1.3
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_C1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_D1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_D1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_D1.1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_E1

East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_F1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_F1.1
East Africa (Tanzania and Kenya)	Cosmopolitan	AF1b	Cosmopolitan AF1b_F1.1.1
Southeast Asia (Philippines)	Asian	SEA4	Asian SEA4_A1
Southeast Asia (Philippines)	Asian	SEA4	Asian SEA4_A1.1
Southeast Asia (Philippines)	Asian	SEA4	Asian SEA4_A1.1.1
Southeast Asia (Philippines)	Asian	SEA4	Asian SEA4_A1.1.2
Southeast Asia (Philippines)	Asian	SEA4	Asian SEA4_A1.2
West Africa (Nigeria)	Africa	Africa 2	Africa 2_A1.1
West Africa (Nigeria)	Africa	Africa 2	Africa 2_B1

3.5 Discussion

In this study, we present an updated, accessible RABV nanopore-based whole genome sequencing workflow, and is the most comprehensive publicly available protocol with complete sample-to-sequence-to-interpretation steps built upon the protocol developed by Brunker et al. (Brunker et al., 2020) using resources from the ARTIC network (Quick et al., 2017). The workflow encompasses the complete process from sample preparation to sequence generation and data interpretation, highlighting two rabies-specific tools to automate lineage assignment and determine phylogenetic context. The enhanced workflow provides comprehensive instructions for preparing brain tissue samples, executing the bioinformatics pipeline, and employing rabies-specific tools for lineage classification and phylogenetic context determination. Primer mismatches are attributed to genetic variability among target sequences, influenced by regional genetic diversity and differences in reference genomes used during primer design. This optimisation enhances sequencing accuracy and supports the identification of diverse RABV lineages. Overall, this analysis allows for optimisation of the primer scheme, enabling more efficient and accurate genomic surveillance of rabies virus, particularly in regions with high viral diversity such as East Africa. We have provided guidance on optimising multiplex primer schemes to improve coverage and address accumulated viral diversity (Supplementary File B.1.2).

We have demonstrated the successful implementation of the workflow in both academic and research institute settings in endemic LMICs with no or limited genomic surveillance capacity. The workflow has proven resilient to application across diverse settings, and comprehensible by users with varying expertise.

The protocol leverages the cost-effectiveness of nanopore sequencing, significantly reducing both startup and operational costs. The time and cost required for library preparation and sequencing with Nanopore is greatly reduced relative to other platforms such as Illumina (Brunker et al., 2020). Additionally, continual technology developments are improving sequence quality and accuracy to be comparable with Illumina (Bull et al., 2020). By optimising the use of flow cells and implementing cost-saving strategies, such as reducing sample number per run (e.g., from 24 down to 12 samples) can extend the life of flow cells over multiple runs. Whereas increasing the number of samples per run will maximize time and reagents. In our hands, we were able to wash and reuse flow cells for one in every three sequencing runs, enabling an additional 55 more samples to be sequenced. Washing the flow cell immediately after use or if not possible, removing the waste fluid from the waste channel after every run, seemed to preserve the number of pores available for a second run. Taking into consideration the initial number of pores available in a flow cell, one run can also be optimized to plan how many samples to run in a particular flow cell.

Efforts have also been made to help users to allow for ease of procurement in a given region, which is typically a challenge for the sustainability of molecular approaches (Okeke & Ihekweazu, 2021). For example, in Africa (Tanzania, Kenya and Nigeria), we opted for Blunt/TA Ligase Master Mix at the adapter ligation step, which was more readily available from local suppliers and a cheaper alternative to other ligation reagents.

This protocol is designed to be resilient in diverse low resource contexts. By referring to the troubleshooting and modifications guidance provided alongside the core protocol, users are supported to adapt the workflow to their needs. The addition of user-friendly and accessible bioinformatic tools represents a major advancement in the original protocol, providing rapid and standardized methods that can be applied by users with minimal bioinformatics experience to analyse and interpret sequencing data in local contexts. The capacity to do this in situ is often limited by the need to have specific programming and phylogenetic skills, which require an intensive and long-term skills training investment. While this skillset is important to thoroughly interpret sequence data, basic and accessible interpretation tools are equally desirable in order to capacitate local “sequencing champions”, whose core expertise may be wet lab based, enabling them to interpret and take ownership over their data. However, hands-on training remains essential for mastering critical steps like SPRI bead clean-ups and minimizing contamination

Contamination has consistently been a major concern when working with amplicon-based sequencing, particularly due to the difficulty of detecting cross-contamination between samples during post-sequencing analysis. While the original discussion acknowledged this challenge, additional insights from implementation have allowed us to refine contamination control measures. Key lessons learned include the importance of maintaining clean workspaces, physically separating pre- and post-PCR areas, and using negative controls in each run. Assigning dedicated pipettes for pre- and post-PCR processes also proved effective in minimizing contamination risks. These practical improvements have been critical to increasing the protocol's reliability across different settings, especially in resource-limited environments, and emphasize the ongoing need for rigorous laboratory practices to ensure data quality.

The fast pace of nanopore sequencing developments is both an advantage and disadvantage for routine RABV genomic surveillance. Continuing improvements to nanopore's accuracy, accessibility and protocol repertoire widen and improve the scope for its application. However, these rapid advancements also risk rendering standard operating procedures and bioinformatic pipelines obsolete. To address this, we provide a transition document to assist in adapting from older to current nanopore library preparation kits (B.1.5). Additionally, regular updates to protocols and continuous engagement with the nanopore sequencing community will help maintain the workflow's relevance and longevity.

A common roadblock to sequencing in LMICs is accessibility, including not only cost but the ability to procure consumables in a timely manner (particularly sequencing reagents, which are relatively new to procurement teams and suppliers), computational resources and simply having access to stable power and internet. Using portable nanopore sequencing technology as the foundation of this workflow helps with many of these accessibility issues and we have demonstrated the use of our protocol across a range of settings, conducting the full protocol and analysis in-country. Admittedly, procuring equipment and sequencing consumables in a timely manner remains a challenge and, in many instances, we were forced to carry or ship reagents from the UK. However, in some areas we were able to rely entirely on local supply routes for reagents, benefiting from investment in SARS-CoV-2 sequencing (e.g., Philippines) that has streamlined procurement processes and begun to normalize the application of pathogen genomics. The variability in sequencing costs across LMIC settings highlights the need for improved local supply chains and infrastructure to enhance the feasibility of in-country genomic surveillance.

The need for a stable internet connection is minimized by one-time-only installs e.g., GitHub repositories, software download, and MinION sequencing itself only requires internet access to start the run (not throughout) or can be performed completely offline with agreement from the company. If mobile data is available, a phone can be used as a hotspot to the laptop to begin the sequencing run, before disconnecting for the run duration. When routinely processing samples, data storage requirements can grow rapidly and ideally data would be stored on a server. Otherwise, SSD hard drives are relatively cheap to source.

While we recognize that there are still barriers to genomic surveillance in LMICs, increasing investment in building genomics accessibility and expertise (e.g. Africa Pathogen Genomics Initiative (Africa PGI)) (Inzaule et al., 2021) suggests that this situation will improve. Genomic surveillance is critical for pandemic preparedness (WHO, 2022), and capacity can be established through routinizing genomic surveillance of endemic pathogens such as RABV. Global disparities in sequencing capacities highlighted during the SARS-CoV-2 pandemic should be a driver of catalytic change to address these structural inequities.

This sample-to-sequence-to-interpretation workflow for RABV, including accessible bioinformatics tools, has potential to be used to guide control measures targeting the goal of zero human deaths from dog-mediated rabies by 2030 and ultimately for the elimination of rabies virus variants. Combined with relevant metadata, genomic data generated from this protocol facilitates rapid RABV characterization during outbreak investigations and in identification of circulating lineages in a country or region (Brunker et al., 2020; Bull et al., 2020; Campbell et al., 2022). We illustrate our pipeline using mostly examples from dog-mediated rabies; however, the workflow is directly applicable to wildlife rabies. This transferability and low cost minimizes the challenges to making routine sequencing easily available not only for rabies but also for other pathogens (Pallerla et al., 2022; Quick et al., 2016), to improve disease management and control.

3.5.1 Limitations of the Study

There are some limitations to this study that may affect how widely and sustainably the workflow can be used. Although the protocol was developed to be user-friendly, especially for those with limited bioinformatics experience, hands-on training is still important.

The rapid pace of updates in nanopore sequencing technology is a challenge every protocol has to undergo. As new versions of reagents and software become available, protocols may

need to be revised often, which could require additional training. Additionally, alternative Nanopore Technology protocol using a different product line like the Flongle was not evaluated in this work which could have offered a cheaper assay for genomic surveillance. Procuring reagents and equipment locally also remains a problem in many LMICs, despite using more accessible alternatives where possible. Internet and data storage limitations can further restrict the ability to process and analyse data fully in-country.

Lastly, while the workflow can be used for both dog-mediated and wildlife rabies, it was mostly tested with dog samples. More work is needed to confirm how well it performs for wildlife cases. Still, this workflow offers a practical and low-cost option for expanding rabies genomic surveillance in resource-limited settings.

Chapter 4

Characterization of the diversity and distribution of Rabies Virus in the Philippines

4.1 Abstract

Genomic data and geographic information combined provide valuable insights into a pathogen's evolution history; when, where, and how it evolves and diversifies. This information is crucial in the management and control of rabies. Here, I analysed the phylogenetic diversity of RABV whole genome sequences collected from 33 of the 81 provinces in the Philippines over 25 years from 1998 to 2023. Using MADDOG, a publicly available tool for classification into phylogenetic lineages, RABV sequences generated from the samples were classified. For phylogenetic inference, I used BEAST and auxiliary programs (Beauti, Tracer, LogCombiner, TreeAnnotator). To account for sampling bias, Bayesian Tip Association Statistics Testing was used to test for strength of association. Whole genome sequencing compared to partial genome sequencing, provides a higher resolution to provide insights into the virus' transmission dynamics at scales relevant to local control. I found that RABV in the Philippines, exhibits strong geographic associations in RABV spread, with clustering according to major island groups- Luzon, Visayas and Mindanao. This was evident from the lineage diversity and the statistical tests of phylogeny-trait associations. My findings revealed structure at the regional and provincial levels in all the island groups. RABV was mostly contained within island groups, with highest RABV genetic diversity in Luzon while Visayas and Mindanao displayed some level of compartmentalization between regions. This information is useful in formulating area-specific rabies control programs, such as stricter border control in island group and coordinated vaccination drives among regions with shared land borders. Future phylogeographic studies with improved sampling will provide deeper insights into the viral movement over time across the country.

4.2 Introduction

Rabies persists as a global problem, primarily affecting low- and middle-income countries (LMICs) in Africa and Asia (WHO, 2018)19/11/2024 22:13:00. Despite ongoing efforts towards rabies control and elimination, the disease continues to cause fatalities, particularly in underserved and remote areas (Hampson et al., 2015). In 2019, India reported the highest number of rabies deaths (5,206), followed by Nigeria (1,295) and Pakistan (1,198) (Gan et al., 2023). The same study ranked the Philippines 7th, with an estimated 200-300 rabies-related deaths annually (Republic of the Philippines Department of Health, 2024).

The Philippines is an archipelagic country located in Southeastern Asia encompassing over 7,600 islands divided into three major island groups: the Luzon group to the north, the central Visayas, and the Mindanao group to the south. Luzon is the largest island, making up a third of the country's land area, and is home to the capital, Manila. Mindanao is slightly smaller, while the Visayas is a discontinuous cluster of smaller islands. The country is divided into 17 regions, which are split into provinces, cities, municipalities, and barangays (Licuanan et al., 2019) (Figure **4.1**). The country is highly diverse, and its complex geographic landscape presents a public health challenge for disease surveillance due to limited healthcare access, delays or inconsistencies in disease reporting, and weaker health systems to enforce quarantine or vaccination programs across islands. However, the archipelagic landscape itself naturally restricts the movement of pathogens (Hemming-Schroeder et al., 2018; Lambin et al., 2010; Morand & Lajaunie, 2019) and can support effective containment strategies in the transmission and spread of infectious diseases like rabies.

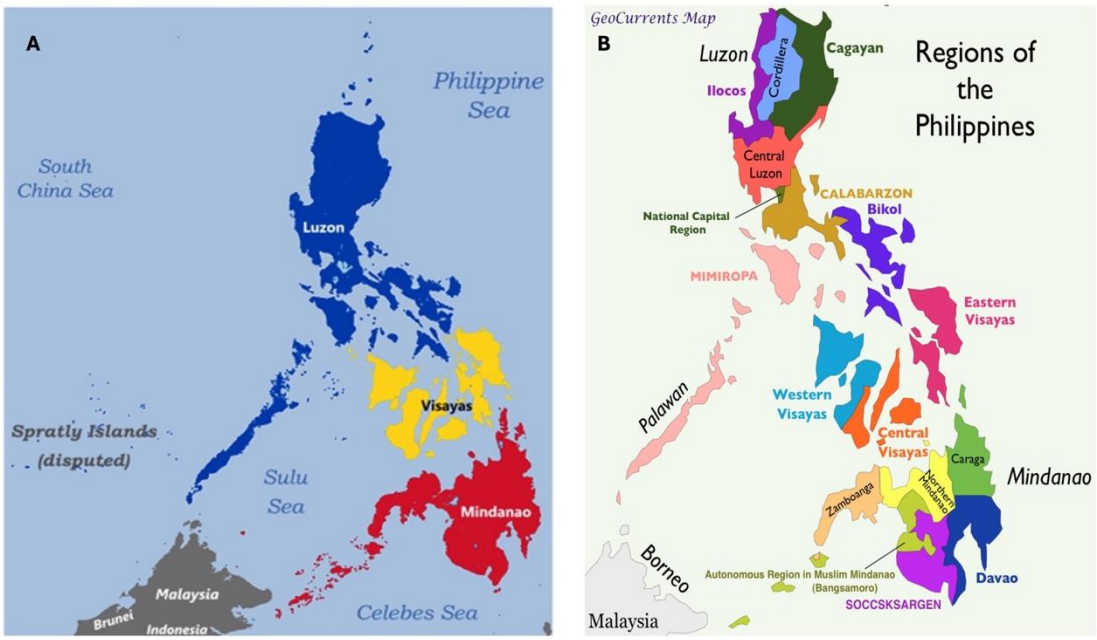

Figure 4.1**. The geographic and administrative structure of the Philippines** A. Map of the Philippines showing the three major island groups: Luzon (blue), Visayas (yellow), and Mindanao (red) bounded by the South China Sea to the west, Sulu and Celebes seas to the south, and Pacific Ocean to the north and east (not listed in the map) **(PhilAtlas, 2025)**. B. Map of the Philippines showing the 17 regions: 8 in Luzon, 3 in Visayas, and 6 in Mindanao .**(Pinterest, 2025)**

The country's rabies control strategy involved establishment of the National Rabies Prevention and Control Committee (NRPCC) in 1991 and further strengthened by the passage of Republic Act No. 9482 (Anti-Rabies Act of 2007), which formalized a multisectoral approach involving national agencies, local government units, and community

stakeholders ((Department of Agriculture et al., 2008). Pursuant to the Global Strategic Plan to end human dog-mediated rabies deaths by 2030 (WHO/FAO/OIE/GARC, 2018), the Philippines has drafted a "National Strategic Plan for Rabies Elimination" which aimed to standardize mass dog vaccination, improve surveillance, and regulate the movement of dogs across regions. Regulatory measures were developed that include registration and vaccination of dogs which entails prior estimation of the dog population and identifying the target population (all healthy dogs aged 3 months and above) for vaccine requirement. Additionally, dog population management was recommended including stray dog management, impounding, and spaying and castration were implemented with enforcement of penalties and fines. Dog movement controls outlined such as control of cross border movement (inter-provincial and inter-island) by requiring certificate of vaccination, health certificate and shipping permit. International dog movement likewise requires clearance to import/export, veterinary health certificate, 30 days quarantine and antibody titre confirmation. However, implementation has varied by region, with areas such as Luzon, particularly Calabarzon and the National Capital Region benefiting from stronger infrastructure and more consistent program coverage, while many parts of Visayas and Mindanao continue to face challenges due to limited veterinary capacity, logistical constraints, and lower prioritization.

To develop more effective control and elimination strategies, a deeper understanding of the Rabies virus's (RABV) genetic diversity and distribution within this varied landscape is needed. Genomic characterization plays a crucial role in elucidating the evolutionary patterns, transmission dynamics, and mechanisms underlying the genetic diversity of RABV. Genome sequences, i.e. the complete genetic material of the virus, provide the resolution required to delineate between closely related cases, such as those occurring during an outbreak, and to measure evolution over observable timescales (i.e. months or years) in relation to ecological and epidemiological processes (Baker et al., 2023; Hill et al., 2023). In-depth phylogenetic analyses can yield quantitative insights into RABV evolution and spread. Phylogeography, for example, enables the investigation of historical processes that influence the geographic distribution of genetic lineages (Kühnert et al., 2011), focusing on their spatial and temporal patterns (Lemey et al., 2009). This field integrates virus genomic data with geographic information to track how, when, and where pathogens move, evolve, and diversify. It can play a crucial role in controlling outbreaks, informing public health decisions, and preparing for future challenges in disease prevention and control.

Advances in high-throughput sequencing technologies and bioinformatic tools now allow for rapid genomic analysis, enabling the characterization of new virus variants within the timeframe of an outbreak (Armstrong et al., 2019). This approach can provide insights into spillover events, mutation rates, and transmission dynamics. For instance, genomic analysis of early viral isolates traced the origins of SARS-CoV-2 to zoonotic transmission, likely from bats, with possible intermediary hosts (Andersen et al., 2020). Similarly, genome sequencing played a key role in tracking viral evolution and transmission chains during the West African Ebola outbreak, helping to inform interventions for future epidemics (Dudas et al., 2017).

In zoonotic diseases like rabies, genomic studies have become invaluable in understanding the genetic diversity and transmission dynamics of the virus. RABV genomic analysis has provided significant insights into the diversity of circulating lineages and their spread among different species, guiding vaccination and control efforts (Brunker et al., 2020). Whole-genome sequencing (WGS) combined with contact tracing has been used to track rabies transmission, supporting a One Health approach that includes vaccination strategies to eliminate rabies, such as from the island of Pemba in Tanzania (Lushasi et al., 2023). In addition, the findings that a strong geographical clustering and limited virus movement between Western and Central Africa are important to be able to design an effective and progressive strategy for rabies elimination in Western and central Africa (Talbi et al., 2009).

In the Philippines, most phylogenetic studies on RABV have used partial genomes, specifically focusing on the Nucleoprotein or Glycoprotein genes (Nishizono et al., 2002; Saito et al., 2013; Tohma et al., 2014). These studies are limited in their ability to capture the full genomic diversity of the potentially missing important variations across the entire genome and hinder the ability to track changes over longer timeframes or across broader geographic regions. This limits the accuracy of evolutionary analyses and constrains wider epidemiological comparisons. However, these studies have provided important insights on RABV emergence in the Philippines. Philippine RABV isolates fall within the major Asian clade and cluster in a distinct minor clade, Asian SEA4 (previously classified as Asian 2b (Gong et al., 2010)), that is specific to the Philippines and distinct from other Asian countries (Nishizono et al., 2002; Saito et al., 2013; Tohma et al., 2014). This Philippines-specific cluster may have been introduced through human-mediated intercontinental translocations from China (Bourhy et al., 2008). Within the country, RABVs are divided into three phylogenetic lineages corresponding to the island groups of Luzon, Visayas, and Mindanao, with sub-lineages found in different geographical locations (Saito et al., 2013; Tohma et al.,

2014). Studies have demonstrated strong regional phylogeographic structure and further clustering within regions (Bacus et al., 2021). However, inter-island transmission has been observed during outbreaks, such as between Luzon and Tablas Islands, likely driven by human movement (Tohma et al., 2016). While this type of transmission appears to be rare, it can have significant consequences leading to the emergence and establishment of rabies in previously free areas (Brunker et al., 2015; Lushasi et al., 2023; Mahardika et al., 2014) (Combining genomics and epidemiology to investigate a zoonotic outbreak: rabies in Romblon Province, Philippines). Understanding these phylogeographic associations is key to determining the best approach for rabies control strategies e.g. targeted, island-specific mass dog vaccination campaigns, and determining the frequency and impact of human-driven transmission as a driver of cross-regional spread.

The Philippines ranks second, after China, in the number of published RABV genomic studies in Asia (Jaswant et al., 2024) Despite this, further investigation into RABV transmission dynamics is needed, with greater spatial and phylogenetic resolution. Ensuring representative sample collection across regions is crucial to providing a broader understanding of RABV genomic epidemiology. As of 2023, public databases contained over 500 partial RABV sequences and only 57 whole genome sequences (WGS), rather than just partial genomes, would capture critical genetic variations over timescales relevant to local control strategies. WGS provides better insights to understand viral evolution, transmission, and response to control measures. This study generated over 200 WGS, spanning 25 years (1998-2023), to identify the temporal patterns of genetic diversity and geographic distribution at a finer scale. Ultimately, the research in this chapter aims to uncover rabies spread and transmission pathways, providing insights to inform control strategies based on phylogenetic analysis.

This chapter applies a combination of phylogenetic, temporal and spatial analyses to address key questions relevant to rabies surveillance and control in the Philippines. Specifically, the analyses aimed to answer the questions: What is the evolutionary history and geographic structure of RABV lineages in the Philippines? What are the temporal patterns of RABV emergence and persistence across different regions? And to what extent does inter-island or regional spread of RABV occur? Through these approaches, I explored patterns of localised transmission or introductions, and how these findings can inform region-specific intervention strategies in line with national rabies control efforts.

4.3 Methods

4.3.1 Sample Selection and Data

This study utilised RABV samples archived at the Research Institute for Tropical Medicine (RITM), constituting samples submitted at RITM and through the Regional Animal Disease Diagnostic Laboratories (RADDL) since 1998. Associated epidemiological metadata, provided by the animal's owner or the bite victim's family was obtained through structured interviews (by RITM and RADDLs) capturing details about the biting animal, victim demographics, case management, and the type and condition of the sample collected. Metadata was stored in the laboratory's database, following the department's data collection and management system. From the archived collection of confirmed rabies cases standardised metadata from the period 1998 to 2023 was compiled and aligned with viable brain samples for sequencing, excluding autolysed or improperly sealed samples. At least one representative brain tissue sample from each province, for each available year and month, was selected, predominantly involving domestic dogs. The selected samples primarily originate from the National Capital Region, neighbouring provinces in Luzon, as well as other regions across the Philippines. A total of 165 archived animal brain samples were processed for sequencing, along with 47 additional animal brain samples and 1 human skin biopsy from an active surveillance program in the MIMAROPA region (as discussed in Chapter 5) deploying Integrated Bite Case Management (IBCM).

4.3.2 Sequencing

The selected samples underwent sequencing at the Advanced Molecular Technology Laboratory (AMTL) at RITM, following a standardized workflow for sample processing, sequencing, and data interpretation, as described in Chapter 3. Consensus sequences generated from the artic-rabv (Brunker, 2020/2022) bioinformatic pipeline underwent quality control before phylogenetic analyses. This involved reviewing mapping statistics including genome coverage and variant calling; sequences with less than 90% genome coverage were not included in the analysis. The consensus sequences were compiled into a single FASTA file and aligned using Multiple Alignment using the Fast Fourier Transform (MAFFT) alignment tool, under default settings (Katoh & Standley, 2013). The graphical viewer Aliview was employed to visualize the multi-sequence alignment, providing a detailed examination of the sequence data to look for gaps and misalignment, which were resolved manually if required. After the quality check, a custom R script identifying the corresponding nucleotide positions of the N and G genes based on the reference genome was

used to extract the partial genomes separately from the whole genome consensus sequences. The resulting gene-specific sequences were compiled into separate FASTA files and underwent the same preparation as the whole genome sequences prior to use in further downstream analysis.

4.3.3 Classification of phylogenetic diversity – clade and lineage assignment

RABV-GLUE (Centre for Virus Research, 2023) was used to curate a dataset of publicly available partial and whole genome RABV sequences from the Philippines. This data was combined with the newly generated WGS through a series of steps to standardise, merge and eliminate duplicates using custom R scripts outlined in Chapter 5 (**Error! Reference source not found.**) and its associated online materials (https://github.com/boydorr/outbreak_romblon_PHL). Specifically, this process involved consolidating sequences from the same isolate (e.g. partial genome sequences from different genes under different Genbank accession IDs) into a unified consensus sequence, standardising location data, and employing a previously established method from Holtz et al. (Holtz et al., 2023) to produce a concatenated sequence alignment encompassing all available sequence data, whether partial or whole genome (henceforth referred to as the full dataset, Table 4.1). Subsequently, this alignment was used as input to MADDOG, a publicly available tool for classification into phylogenetic lineages.

To visualise the spatial distribution of identified lineages within the Philippines, shapefiles of administrative boundaries (municipality, provinces and regions) were obtained from the Philippine Standard Geographic Code (PSGC) (https://github.com/altcoder/philippines-psgc-shapefiles) and integrated with sequence metadata using R scripts executed in R Studio (R Core Team, 2021). Lineage cluster maps were generated by overlaying phylogenetic lineage assignments onto the geographic coordinates of sample origin. The same shapefiles were also used in other maps generated for this chapter.

In MADDOG (Campbell et al., 2022), a new lineage is defined by at least one shared mutation that distinguishes it from the ancestral sequence. At least 10 descendants are required to ensure accurate lineage designations and minimize incorrect designation as a result of sequencing errors. Established global RABV phylogenetic groupings from RABV-GLUE served as baseline classification (e.g. Cosmopolitan Af1b is a global major/minor clade designation, see Figure **4.2**) while subsequent MADDOG lineage designations start with lineage A1, following the dynamic lineage nomenclature advised by Rambaut et al

(Rambaut et al., 2020), which was employed for SARS-CoV-2. Descendants to this lineage are labelled A1.1, A.1.2, and so on, while their further descendants become A1.1.1, A1.2.1. After three iterations, a lineage becomes a new major lineage, such as A1.1.1.1 becoming B1 (Figure **4.2**).

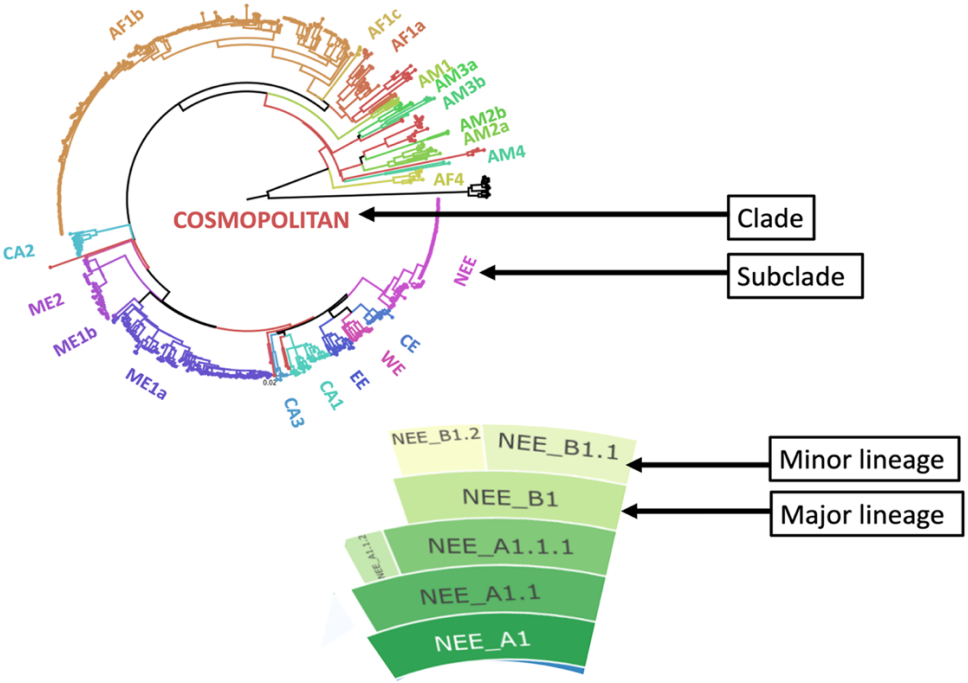

Figure 4.2**. Example of a MADDOG lineage classification showing hierarchical relationships of viruses.** Example of a MADDOG lineage classification showing hierarchical relationship. A Major lineage is designated by a letter and can be either the initial iteration of a lineage or one that has significantly evolved into a new major lineage. A Minor lineage, indicated by numbers (e.g., A1.1.1), represents a lineage that has evolved but not enough to qualify as a new major lineage. Figure reproduced with permission from Campbell et al, 2022

4.3.4 Bayesian Phylogenetic Reconstruction

To investigate the evolutionary history of RABV in the Philippines, I performed Bayesian Markov Chain Monte Carlo (MCMC) analyses of WGS from the study (n=208) and all RABV WGS publicly available from GenBank (n=91) -henceforth referred to as the WGS dataset (Table 4.1)- using BEAST v1.10.4. In addition to the WGS dataset, separate BEAST analyses were conducted on the N and G gene segments extracted from the same set of whole genome sequences to compare phylogenetic resolution and temporal estimates. This comparison aimed to evaluate the extent to which partial gene sequences, commonly used in historical datasets, capture similar evolutionary and transmission patterns as full genomes.

4.3.4.1 Assessing temporal signal

To assess the temporal signal in the WGS dataset, a root-to-tip regression analysis was conducted using Tempest v1.5.3. The maximum likelihood (ML) phylogenetic tree was first reconstructed in IQ-TREE. The R-squared value from the linear regression was used to quantify the strength of the clock-like signal.

4.3.4.2 Model selection

Before BEAST analysis, ModelFinder (Campbell et al., 2022; Kalyaanamoorthy et al., 2017) was implemented in IQ-TREE (Nguyen et al., 2015) to identify the optimal substitution model for the dataset. Using this process I also assessed whether genome partitioning by gene or coding/non-coding region was necessary by utilising partition models (Chernomor et al., 2016). To facilitate this a partition configuration file was created to divide the sequence data based on codon positions (CP): 1st, 2nd, and 3rd, as well as gene/non-coding regions in the genome. This partition file was used as input to IQ-TREE, along with the sequence alignment, to determine the best-fit partitioning scheme and associated optimal substitution models. ModelFinder aims to merge positions effectively to avoid over-parameterization and improve model fitting. The optimal scheme, as determined by the Bayesian Information Criterion (BIC) involved separate partitions for concatenated coding and non-coding regions. For the coding region, the scheme included two codon positions (CP112), utilising the General Time Reversible substitution model with empirical base frequencies and rate heterogeneity using a Gamma distribution (GTR + F + G). For the non-coding partition the same model, without codon partitioning, was applied.

4.3.4.3 BEAST analysis

The substitution model identified above was used, along with a Bayesian skyline model with 10 groups as a coalescent tree prior to estimating population changes over time. The skyline model was employed to allow flexibility in population sizes over time intervals, avoiding strong assumptions of constant or exponential population growth. To enable model comparison of different molecular clock models (Table 4.2) the marginal likelihood was estimated using BEAST's path sampling (PS) and stepping stone (SS) methods (Baele et al., 2012; Xie et al., 2011). Two independent MCMC chains were run for 500 million states and 800 million states, sampling every 50,000 and 80,000 states respectively. Runs were checked for adequate mixing and convergence in Tracer v1.7.1 (Rambaut et al., 2018) alongside model comparison with PS/SS results. Independent runs obtained from the best model were

then combined and the maximum clade credibility tree (MCC) was generated using TreeAnnotator v1.10.4, discarding the first 10% of the chain as burn-in. Tree annotation was conducted in FigTree v1.4.4.

4.3.5 Bayesian Tip-association Significance Testing

Bayesian Tip-association Significance Testing (BaTS) (Parker et al., 2008) was used to quantify phylogeny-trait associations, in this case, to assess how phylogenetic relatedness correlates with geographic clustering. This method considers the uncertainty arising from phylogenetic error by using a posterior set of trees generated by BEAST and provides statistical significance tests of the null hypothesis that traits are associated randomly with phylogenetic tips. This is done by statistically testing the observed trait distribution against what would be expected by chance. The latest version of the BaTs software, Befi-BaTs v0.10.1, was used to provide several statistics to assess phylogeny-trait associations:

1) Association index (AI) is a test statistic that measures whether closely related taxa share traits more often than expected by chance accounting for shared ancestry. A value closer to 0 indicates a high population subdivision.

2) The parsimony score (PS) measures the minimum number of changes required to explain the observed data. Lower PS values indicate stronger geographic clustering of viral lineages.

3) Monophyletic clade size (MC) is the number of lineages within a monophyletic group. Larger monophyletic clades may indicate more stable transmission dynamics or ecological niches, and smaller ones suggest recent divergence or unique transmission events.

4) Unique Fraction Metric (UniFrac) is used to quantify the phylogenetic distance between different groups based on unique and shared lineages. Higher values indicate greater genetic differentiation between groups.

Using this method I assessed geographic clustering at varying resolutions, ranging from coarse to fine levels, from major island groups (n=3) to regions (n=13), and provinces (n=33).

4.3.6 Continuous Phylogeographic Analysis

To explore the spatiotemporal spread of RABV in the Philippines, a continuous phylogeographic analysis was conducted using BEAST v1.10.4. Only WGS (n=292) with known sampling dates and geographic coordinates at the city or municipality level were included to ensure spatial resolution suitable for continuous trait inference (Table 4.1). The GTR + F + Gamma substitution model was used along with a relaxed uncorrelated gamma molecular clock. A Bayesian Skyline model was selected as the tree prior. Two independent MCMC chains were run for 500 million steps, sampling every 50,000 steps. Runs were combined using LogCombiner v1.10.4 after removing 10% burn-in, ensuring effective sample size (ESS) of >200 for key parameters. The MCC tree was summarised using TreeAnnotator and visualised in Spread.GL, a web-based tool for continuous phylogeography.

4.4 Results

4.4.1 Sequencing results

Of the 213 sequenced samples, 208 had 90% or higher genome coverage and were included in the analysis. Most samples came from dog brains (202), with 5 from cats, and one from a cow. Samples (Table C. 1) were collected from 33 of the 81 provinces in the Philippines from 1998 to 2023. An additional 408 partial and 91 whole-genome sequences from the Philippines were sourced from RABV-GLUE, resulting in a dataset of 707 sequences (Table 4.1).

Table 4.1. Summary of dataset and sequence types used in phylogenetic analyses

Dataset	Analysis	Number and Type of sequences	Source
Full (with contextual sequences)	MADDOG lineage assignment (4.3.3)	Partial genome = 408 WGS= 299 Total=707	This thesis=208 RABV-GLUE= 615
WGS	Bayesian Phylogenetic Reconstruction (4.3.4.3; BaTS (4.3.5)	WGS = 299 Total =299	This thesis = 208 NCBI GenBank = 91
	Continuous Phylogeographic Analysis (4.3.6)	WGS = 292 Total =292	This thesis = 208 NCBI GenBank = 84
N gene and G gene	Bayesian Phylogenetic Reconstruction (4.3.4.3)	N gene = 299 G gene =299	This thesis = 208 NCBI GenBank = 91

4.4.2 Circulating lineages

Using the MADDOG classification of phylogenetic lineages, analysis of the full dataset of RABV sequences in the Philippines identified three major lineages: A1, B1, and C1. These lineages are descendants of the Asian SEA4 major/minor clade (Figure 4.3A). The earliest lineage, Asian SEA4_A1, was first detected in samples from 1998 and persisted until 2019. Over time, this lineage diversified into Asian SEA4_A1.1, eventually giving rise to the most recent lineage, Asian SEA4_C1.1. which was detected starting 2007 and remained present up to 2022 (Figure 4.3B).

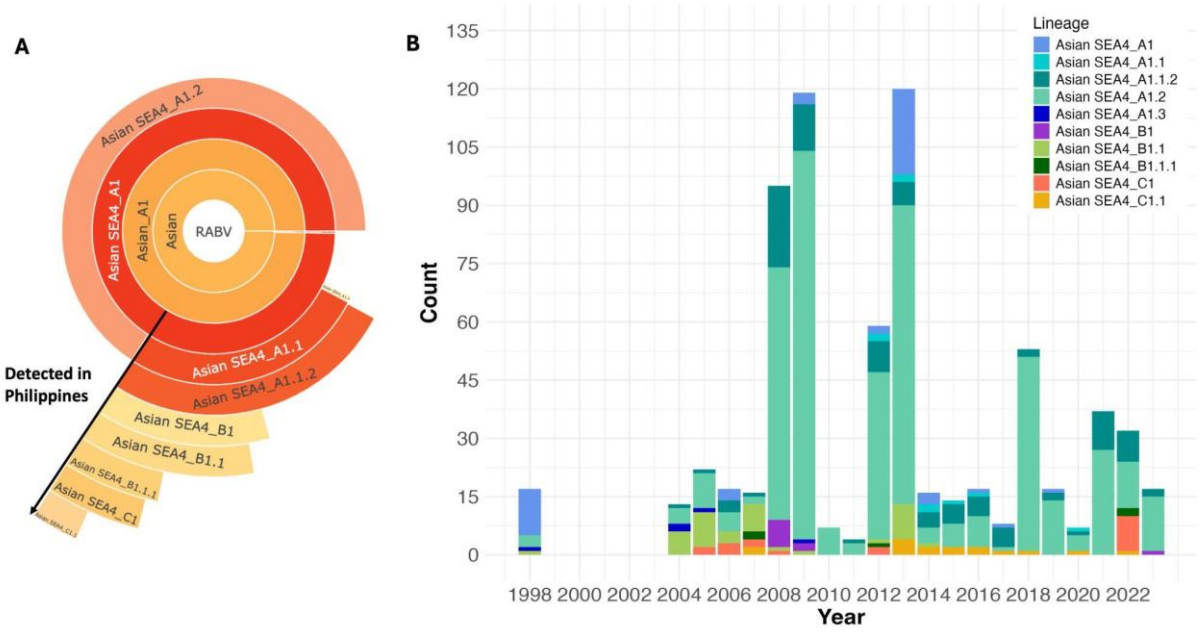

Figure 4.3.RABV lineage diversity through time.

A. Sunburst plot showing the MADDOG-assigned hierarchical lineage relationships, tracing global ancestry to the most recent RABV descendants detected in the Philippines, with distinct shades representing different lineages. B. Lineages observed in the Philippine samples per year from 1998 – 2023.

Among the ten lineages, lineage Asian SEA4_A1.2 has been the predominant lineage circulating in Luzon and is the only lineage circulating in Mindanao and the Visayas over the observed time period (Figure **4.4**A). Only in Luzon is there evidence of the diversification of lineages beyond A1.2, with all ten detected lineages found circulating there. The provinces of Cavite, Batangas, Laguna, located within the Calabarzon region (also known as Region 4A), and Metropolitan Manila from the National Capital Region (NCR) have the highest diversity, each hosting seven lineages over the years (Figure **4.4**). Minor lineage Asian SEA4_B1 and its descendants were mainly found in samples from NCR, Calabarzon, Central Luzon, and Cordillera Administrative Region (CAR), including the only human sample from the Southwestern Tagalog Region (Region 4B) classified as lineage Asian SEA4_B1. In contrast, the most recent lineages, Asian SEA4_C1 and Asian SEA4_C.1.1, were detected in Region 4A, 4B, and NCR (Figure **4.4**). However, Luzon regions have a disproportionately higher number of samples compared to the rest of the regions in the country (Figure **4.4**A). The Visayas region has the fewest samples collected in the dataset (n=6)- representing only 5 of its 16 provinces, while Mindanao has 65 samples from 10 of its 23 provinces.

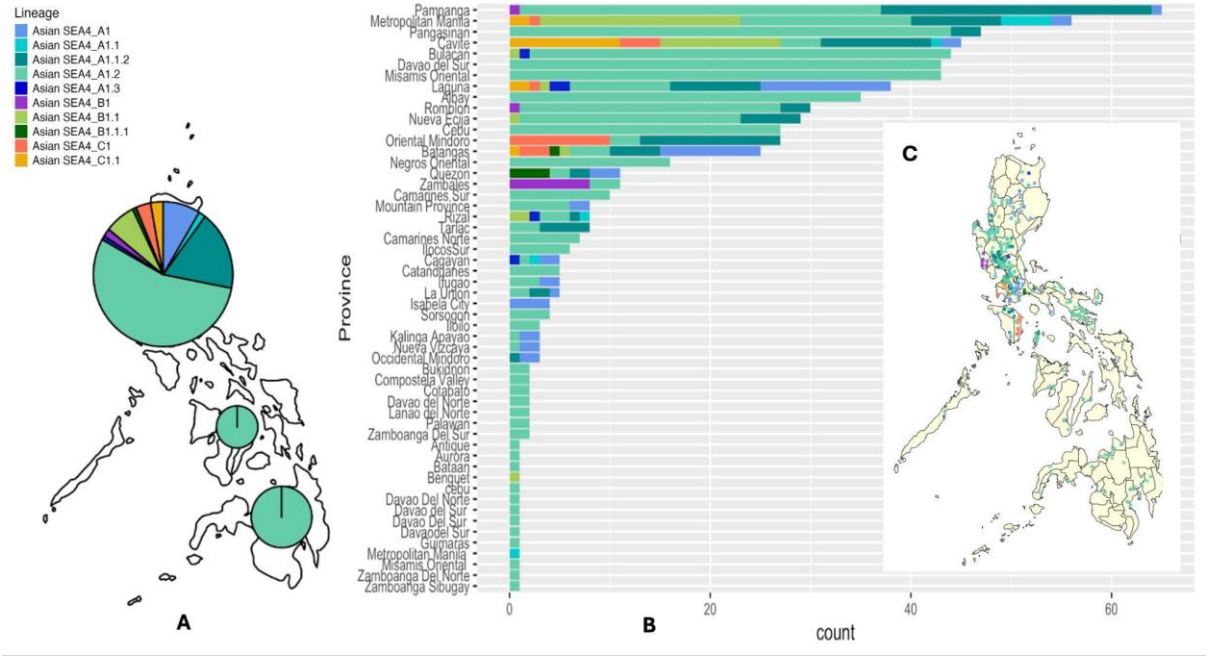

Figure 4.4**. Spatial distribution of RABV lineages across the Philippines.** A. Map showing the proportion of lineages in each island group. B. Bar graph showing the lineage assignments of sequences from each province. C. Map of Philippines provinces showing placement of samples coloured by lineage

The ML tree constructed from the full dataset reveals distinct phylogenetic clustering defined by island groups: Luzon (two clusters: Luzon 1 and Luzon 2), Visayas, and Mindanao (Figure **4.5**A). Although the predominant lineage, Asian SEA4_A1.2, is found nationwide, island-specific clustering within this lineage is still observed in the tree. Luzon1 comprises mainly sequences from the earliest samples, which belong to Asian SEA4_A1, the basal lineage in the Philippines (Figure 4.3A), suggesting Luzon was the point of emergence of RABV in the Philippines. Luzon2 is the largest phylogenetic grouping in the tree, further dividing into two clusters: one consists solely of sequences from Asian_SEA4_A1.2, including samples mostly from northern Luzon, while the second cluster contains the most recent lineages, which were predominantly found in the southern regions, and exhibits the highest lineage diversity observed in the entire phylogenetic tree (Figure **4.5**A). Despite very strong island group associations, several instances of cross-island transmission are evident from the tree: two Luzon samples grouped within the Visayas cluster, while one Visayas sample was detected in Luzon, and another Luzon sample grouped within the Mindanao lineage (Figure **4.5**A).

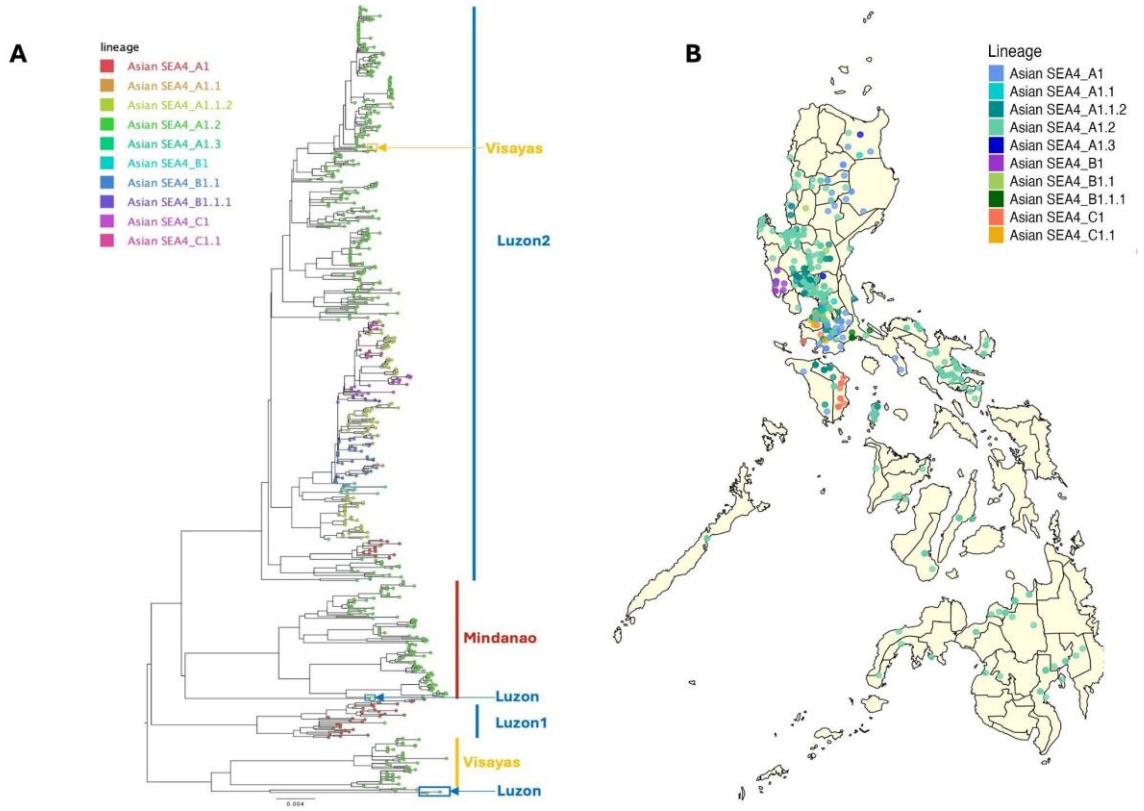

Figure 4.5**. RABV Lineage Clusters within the Philippines.** A. Maximum Likelihood tree of partial (n= 408) and whole genome (n=299) RABV sequences in the Philippines showing placement of lineages clustered in each island group; Luzon, Mindanao, and Visayas with Luzon2 having the greatest number of lineages circulating. Samples that are grouped with another island group are boxed within the phylogenetic tree. The scale shows the substitution per site per year at 0.004. B. Map showing provinces where samples are collected from, with samples coloured by lineage.

4.4.3 Temporal signal

The analysis of 299 WGS, consisting of 208 from this study and 91 other WGS from the Philippines available in GenBank showed a good temporal signal yielding an R^2 value of 0.5025, indicating that the dataset has a moderate correlation suitable for molecular clock-based analysis. There were no apparent outliers in the root-to-tip regression plot (Figure 4.6), indicating that all sequences could be reliably included in further evolutionary analyses without the need to remove any potentially problematic sequences. The variation around the regression line in the root-to-tip regression plot suggested that a relaxed clock model, rather than a strict clock model, is more appropriate for this data.

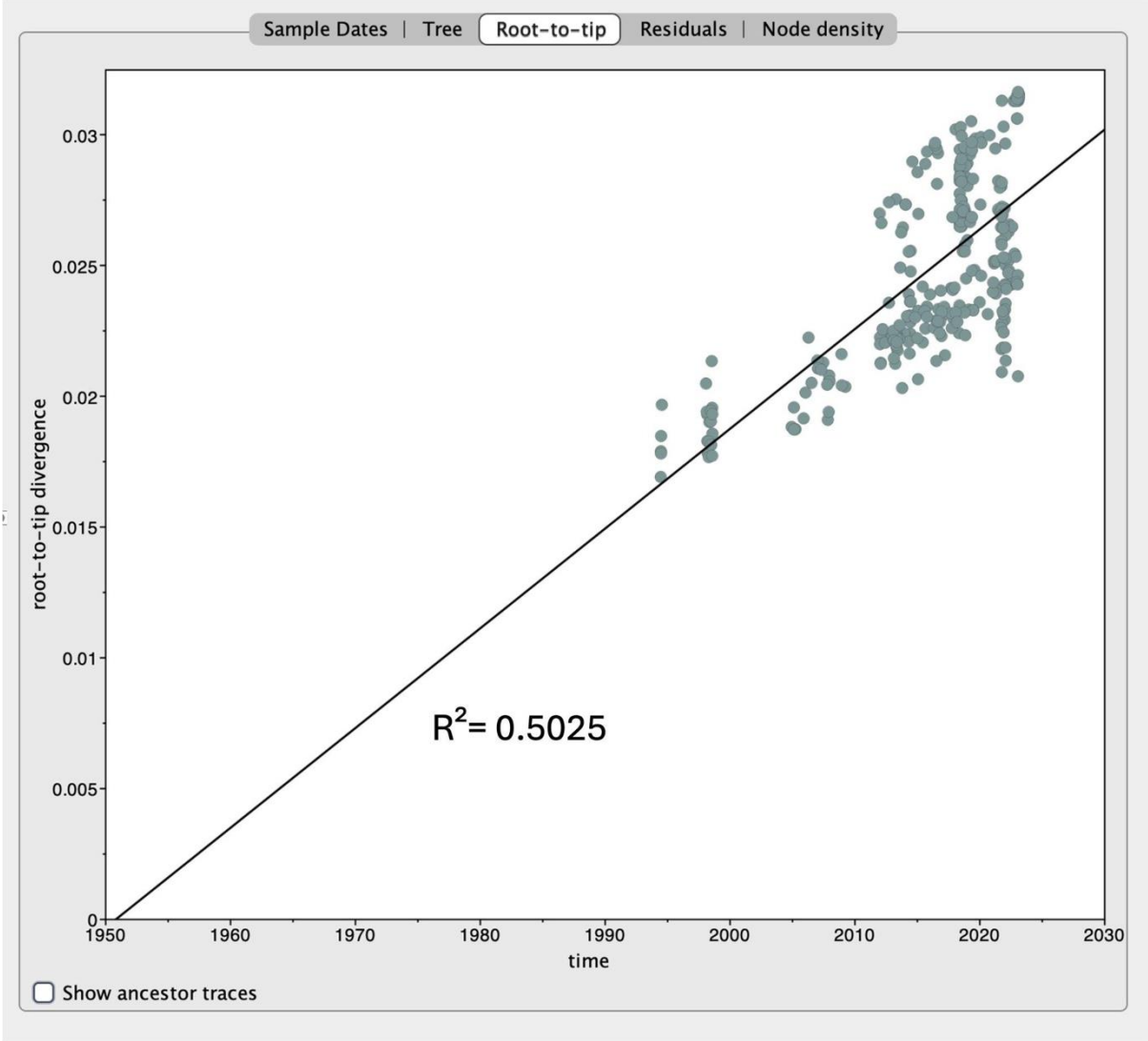

Figure 4.6. Root-to-tip regression analysis of 299 WGS from the Philippines using Tempest. The x-axis represents the sampling dates, and the y-axis represents the root-to-tip genetic divergence from the ML phylogenetic tree.

4.4.4 Molecular Clock comparisons

A comparison of different relaxed molecular clock models showed the strongest overall support for the uncorrelated relaxed clock model with underlying gamma distribution (UCGD), which had the highest marginal likelihood (path sampling (PS) and stepping stone (SS) value) alongside good model mixing and convergence at effective sample size (ESS) >200 (Table 4.2). While the PS/SS results alone favoured a different model - the uncorrelated relaxed clock model with underlying lognormal distribution (UCLD)- this model showed poor convergence, limited mixing, and low ESS values when reviewed in Tracer. Overall, longer MCMC chains were required (800 million rather than 500 million) in order to achieve convergence for any of the models.

Table 4.2. Comparative Analysis of Molecular Clock and Partition Models. Marginal Likelihood Estimates are reported from Path Sampling (PS) and stepping stone (SS) sampling in BEAST.

Codon Partition Model	**Model Description**	**Molecular Clock Model**	**Length of MCMC chain**	**PS Marginal Likelihood**	**SS Marginal Likelihood**	**ESS**	**Notes**
CP 112	Sequence partitioned into: 1 concatenated coding region (5 genes) with codon partitioning and 1 concatenated non-coding region. Codon partitioning: partitioned into codon positions 1&2, 3	***Uncorrelated Relaxed clock model with underlying gamma distribution (UCGD)**	500M	-64370.09	-64385.39	>200	good convergence on most parameters except the skyline group size
			800M	**-64254.70**	**-64272.85**	**>200**	good convergence on most parameters except the skyline group size
		Uncorrelated Relaxed clock model with underlying lognormal distribution (UCLD)	500M	-64245.28	-64259.67	< 200	didn't converge on most parameters and most ESS values are low
			800M	-64263.99	-64282.44	>200	good convergence on most parameters except the skyline group size
		Uncorrelated Relaxed clock model with underlying exponential distribution (UCED)	500M	-64287.82	-64300.54	<200	converged; but only in some parameters and most ESS values are low
			800M	-64294.00	-64310.48	>200	good convergence on most parameters except the skyline group size

No partition	Alignment as whole genome sequence No codon partitions	UCGD	500M	-66552.75	-66572.70	>200	good convergence on most parameters except the skyline group size, low ESS value in the treeLength parameter
		UCLD	500M	-66581.11	-66598.68	<200	good convergence on most parameters except the skyline group size, low ESS value in mean rate, and treeLength parameters
		UCED	500M	-66608.64	-66627.75	<200	good convergence on most parameters except the skyline group size, low ESS value in mean rate, and treeLength parameters

*Final molecular model used in BEAST analysis

4.4.5 BEAST Phylogenetic analyses

The Maximum Clade Credibility (MCC) tree illustrates the evolutionary relationships of RABV WGSs in the Philippines (Figure 4.7A), including estimates of the divergence times of ancestral nodes. The overall tree structure had strong posterior support, indicating that the inferred evolutionary patterns are well supported by the data. The time of the most recent common ancestor (tMRCA) was estimated to be 1932, with a 95% Highest Posterior Density (HPD) range from 1904 to 1956. The phylogeny splits into four clades aligning with major island groups: Luzon, Visayas, and Mindanao, with Luzon further divided into two distinct clades (as also seen in the substitution-only tree presented in Figure **4.5**A). Most clades are well supported in the tree, except the Visayas clade, which has a posterior support value of less than 50%. The Luzon1 clade, the oldest, diverged around 1970. The Visayas clade emerged in early 1975 followed by the Luzon2 lineage in late 1975. The youngest clade, Mindanao, diverged around 1980, sharing a common ancestor with Luzon2. Inter-regional clustering within island groups showed strong geographic containment of viral lineages. In Luzon, viruses found in Central Luzon, Bicol, and Region 4B form distinct clusters, while viruses from neighbouring regions like NCR and Region 4A showed more mixing. In the Visayas, despite limited samples, viruses from Western and Central Visayas retained distinct clusters with some shared viral lineages. Mindanao exhibited the strongest regional clustering, with a clear separation of viral lineages between Northern Mindanao, Davao, and Bangsamoro. Occasional mixing across major island groups such as between Luzon and Visayas (Figure 4.7) suggests long-range transmission events.

While the overall clade structure remained largely consistent across all trees, the MCC tree generated from WGS data (Figure 4.7A) provided greater phylogenetic resolution and stronger posterior support compared to trees derived from using the G gene (Figure 4.7B) and N gene (Figure 4.7C). The G and N gene trees showed reduced resolution, particularly for recent divergence events and in clades with limited sequence variation. The estimated time to tMRCA also varied between datasets. The WGS-based tree had a relatively low standard deviation (SD = 13.8) indicating a precise temporal estimate. In contrast, the G and N gene datasets produced slightly older tMRCA estimates - 1942 (95% HPD: 1901-1065) for the G gene and 1944 (95% HPD: 1895-1969) for the N gene – with substantially higher standard deviations and broader HPD intervals, reflecting greater uncertainty in estimating divergence times.

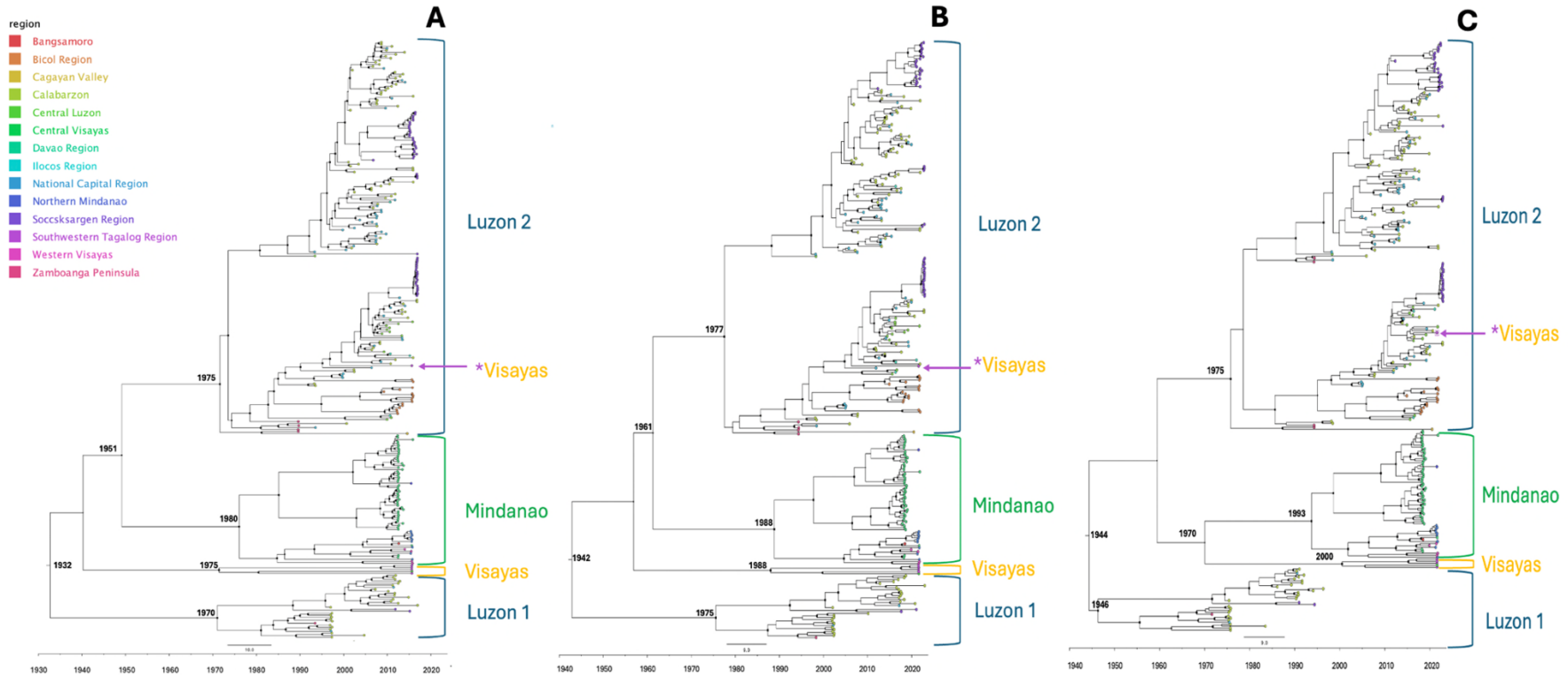

Figure 4.7. Maximum clade credibility (MCC) tree of RABV sequences from the Philippines.

The tree depicts RABV evolutionary relationships and phylogeographic structure. Tips of the tree were coloured according to the region where the samples were collected and major clades Luzon1 (blue), Visayas (yellow), Mindanao (yellow) and Luzon2 (blue) are marked at the side of the tree. Nodes with high posterior probability (>0.5) are represented by squares. The x-axis represents time in years from 1930 to 2025. One sample isolated from Visayas, pointed in purple arrow clustered with Luzon 2 clade. A.) MCC of RABV whole genome sequences B.) MCC of RABV G gene sequences C.) MCC of RABV N gene sequences

The skyline plot (Figure **4.8**) shows a gradual increase in the effective population size around the mid-1980s, which was sustained until the early 2000s. After 2010, the plot shows significant fluctuations, with a sharp increase followed by a steep decline in the population size near the 2020s. The shaded area (blue) around the line represents the confidence interval for the estimates, showing uncertainty in the inference population size. Uncertainty narrows towards the present but widens in the distant past, indicating higher uncertainty in historical population estimates.

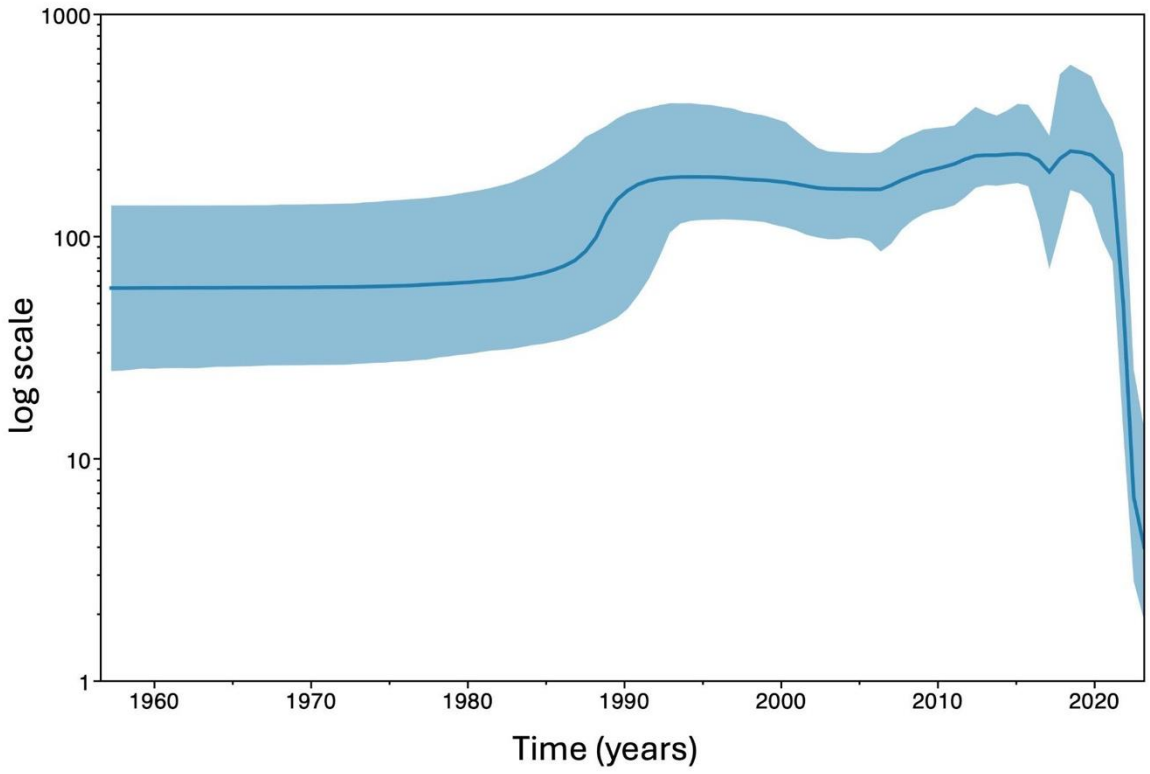

Figure 4.8**. Bayesian Skyline Plot of Effective Population Size Over Time.** The x-axis represents time in years, and the y-axis shows the log-transformed effective population size. The solid line indicates the median population size estimate, while the shaded area represents the 95% Highest Posterior Density (HPD) interval, illustrating the uncertainty in the estimates.

4.4.6 BaTS analysis

Along with the spatial structure observed in the phylogenetic tree (described above), quantitative measures of phylogeny-trait associations using BaTS analysis revealed a strong phylogeographic structure across all geographic resolutions (island, region, and province). This is evidenced by significant P-values <0.05 for the association index (AI), parsimony score (PS), maximum clade (MC), and UniFrac statistics (Table 4.3). The AI results show a weakening of geographic structure at finer resolutions, decreasing from island groups (AI =

9.09E-13) to regions (AI = 5.150) and provinces (AI = 9.013). However, all values remain statistically significant. The PS for island groups (PS = 3.613) is low compared to regions (PS = 75.418) and provinces (PS = 122.619), indicating greater geographic diversity within regions and provinces, but the significant p-values (all <0.001) confirm geography's role in explaining the genetic diversity of RABV. The decreasing UniFrac values from island groups to regions and provinces highlight a lower level of differentiation at finer geographic levels, suggesting more viral movement and transmission occurring more freely at the provincial level within regions. MC statistics were observed to be significant for most regions and provinces except where sample sizes were low. Particularly strong clade differentiation (high MCs) was observed in certain regions e.g. Calabarzon (97 sequences, MC = 7.648), and Davao (50 sequences, MC = 21.000). The following statistics reflect a sampling bias towards Luzon, which accounts for 75% (223/299) of all samples, with 74% (164/223) of these coming from Southern Luzon. Moreover, the Luzon samples cover the widest temporal range, spanning from 1998 to 2023.

Table 4.3. Results of Bayesian Tip-association significance testing (BaTS). Results of BaTS on the genetic diversity of RABV in the Philippines, categorized by geographical location (island group, region, and province). The analysis includes the Association Index (AI), Parsimony Score (PS), Unique Fraction Metric (Unifrac)

Statistic	No. of Sequences	Observed mean (95% CI)	p-value
Island Group			
AI		9.09E-13 (1.63E-18, 1.97E-12)	< 0.001
PS		3.613 (3.000, 4.000)	< 0.001
Unifrac		0.924 (0.899, 0.942)	0.010
Luzon	228	108.389 (108.000, 112.000)	0.010
Mindanao	65	65.000 (65.000, 65.000)	0.010
Visayas	6	4.998 (5.000, 5.000)	0.010
Region			
AI		5.150 (4.774, 5.543)	< 0.001
PS		75.418 (71.000, 80.000)	< 0.001
Unifrac		0.476 (0.438, 0.511)	0.010

Bicol Region	16	14.000 (14.000, 14.000)	0.010
Davao Region	50	21.000 (21.000, 21.000)	0.010
Bangsamoro	2	1.000 (1.000, 1.000)	1.000
Calabarzon	97	7.648 (7.000, 10.000)	0.010
Southwestern Tagalog	51	25.000 (25.000, 25.000)	0.010
unknown	5	1.000 (1.000, 1.000)	1.000
National Capital Region	41	5.000 (5.000, 5.000)	0.010
Central Luzon	13	7.932 (7.000, 8.000)	0.010
Ilocos Region	4	2.000 (2.000, 2.000)	0.020
Northern Mindanao	9	6.071 (6.000, 7.000)	0.010
Cagayan Valley	1	1.000 (1.000, 1.000)	1.000
Western Visayas	5	3.000 (3.000, 3.000)	0.010
Central Visayas	1	1.000 (1.000, 1.000)	1.000
Zamboanga Peninsula	4	2.000 (2.000, 2.000)	0.010
Province			
AI		9.013 (8.473, 9.567)	< 0.001
PS		122.619 (118.000, 127.000)	< 0.001
Unifrac		0.308 (0.279, 0.335)	0.010
Albay	11	6.000 (6.000, 6.000)	0.010
Davao del Sur	45	21.000 (21.000, 21.000)	0.010
Compostela Valley	2	2.000 (2.000, 2.000)	0.020
Davao del Norte	3	1.000 (1.000, 1.000)	1.000
Cotabato	2	1.000 (1.000, 1.000)	1.000
Batangas	18	2.990 (3.000, 3.000)	0.010
Cavite	33	3.687 (3.000, 6.000)	0.050
Quezon	9	2.000 (2.000, 2.000)	0.030
Oriental Mindoro	24	9.674 (9.000, 10.000)	0.010

Romblon	24	20.000 (20.000, 20.000)	0.010
Occidental Mindoro	3	2.000 (2.000, 2.000)	0.010
unknown	5	1.000 (1.000, 1.000)	1.000
Metropolitan Manila	41	5.000 (5.000, 5.000)	0.010
Laguna	30	5.031 (5.000, 5.000)	0.010
Pampanga	3	2.202 (2.000, 3.000)	0.010
Pangasinan	4	2.000 (2.000, 2.000)	0.010
Rizal	7	3.000 (3.000, 3.000)	0.010
Bulacan	6	2.037 (2.000, 2.000)	0.020
Bukidnon	2	1.000 (1.000, 1.000)	1.000
Misamis Oriental	7	2.564 (2.000, 4.000)	0.070
Cagayan	1	1.000 (1.000, 1.000)	1.000
Neuva Ecija	3	1.000 (1.000, 1.000)	1.000
Camarines Norte	2	2.000 (2.000, 2.000)	0.010
Sorsogon	1	1.000 (1.000, 1.000)	1.000
Camarines Sur	2	1.000 (1.000, 1.000)	1.000
Iloilo	3	3.000 (3.000, 3.000)	0.010
Antique	1	1.000 (1.000, 1.000)	1.000
Guimaras	1	1.000 (1.000, 1.000)	1.000
Cebu	1	1.000 (1.000, 1.000)	1.000
Zamboanga del Sur	2	2.000 (2.000, 2.000)	0.010
Zamboanga Sibugay	1	1.000 (1.000, 1.000)	1.000
Zamboanga del Norte	1	1.000 (1.000, 1.000)	1.000
Bataan	1	1.000 (1.000, 1.000)	1.000

4.4.7 Continuous Phylogeographic Analysis

Using a continuous phylogeographic approach, the spatial structure of RABV was further examined, which revealed a predominantly clustered distribution of sequences from the same geographical location, interspersed with a few sequences that originated from distant locations. The overall spread followed a north-to-south trajectory, with RABV initially introduced to Luzon and then spreading locally within island groups with a few long-distance dispersal events from Luzon to Visayas (Figure **4.9** B), and subsequently from Luzon to Mindanao (Figure **4.9** C). An additional long-distance dispersal event from Luzon to the Visayas was inferred to have occurred between 2000 and 2010 (Figure **4.9** G-H), highlighting episodic but significant inter-island transmission events. By the 1990s, localised transmissions within island groups are happening. In the last 10 years from 2010, RABV has been expanding into more provinces within Mindanao (Figure **4.9** H-J).

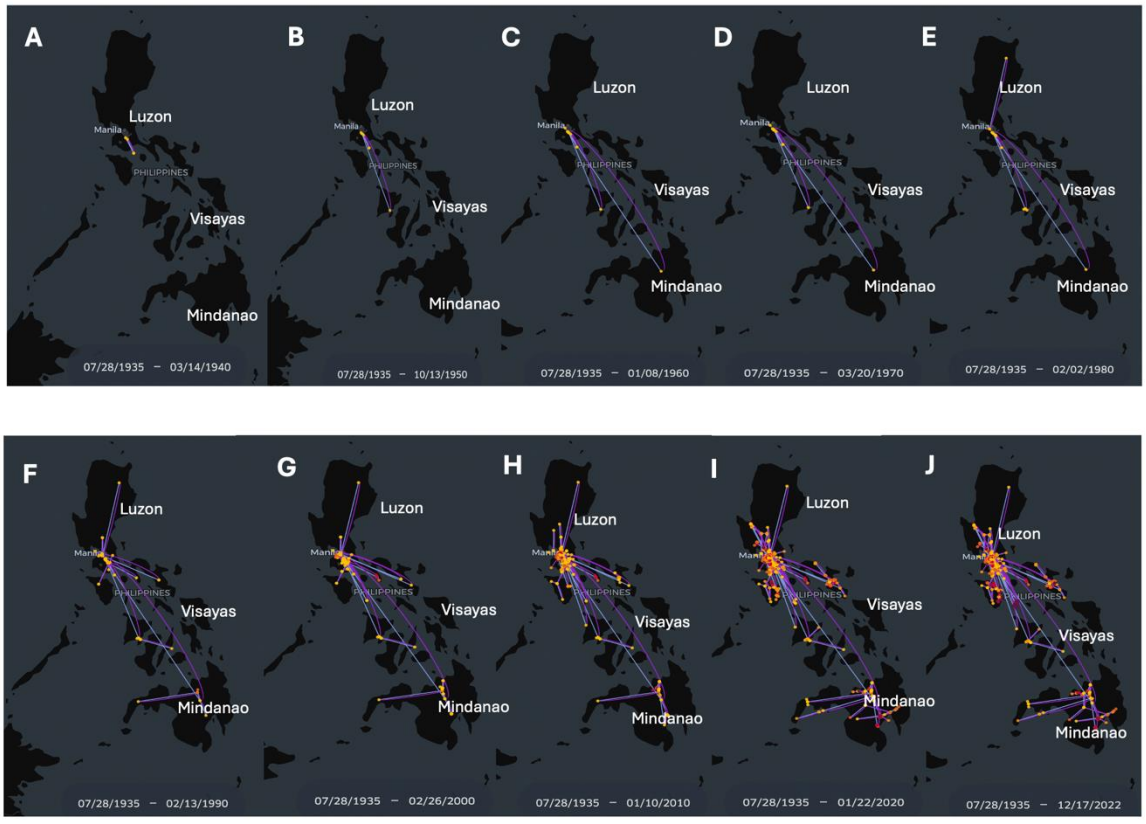

Figure 4.9**. Spatiotemporal Spread of RABV Sequences in the Philippines visualized using Spread.gl.** Spatiotemporal Spread of RABV Sequences in the Philippines visualized using Spread.gl. Phylogeographic reconstruction showing A. Initial introduction of RABV in Luzon island (1930s), B. Southward expansion to the Visayas (1950s), and C. Subsequent spread to Mindanao. By the 1990s, further dissemination occurred across the three major island groups, including localised transmission between provinces within Mindanao.

The phylogenetic tree (Figure **4.10**) revealed the earliest emergence of RABV in Luzon, specifically in the Calabarzon region, with the time to the most recent common ancestor (tMRCA) estimated at 1937 (95% HPD 1906-1956). This pattern aligns with the inferred dispersal pattern shown in the maps expanding to Visayas first with the tMRCA of 1977, followed by divergence to Mindanao in 1980, both originating from Luzon. Notably, the node supporting the Visayas cluster has a low posterior value of 0.27, indicating low statistical support, while nodes along the Luzon and Mindanao branches show higher posterior values, suggesting more reliable phylogenetic relationships in those regions.

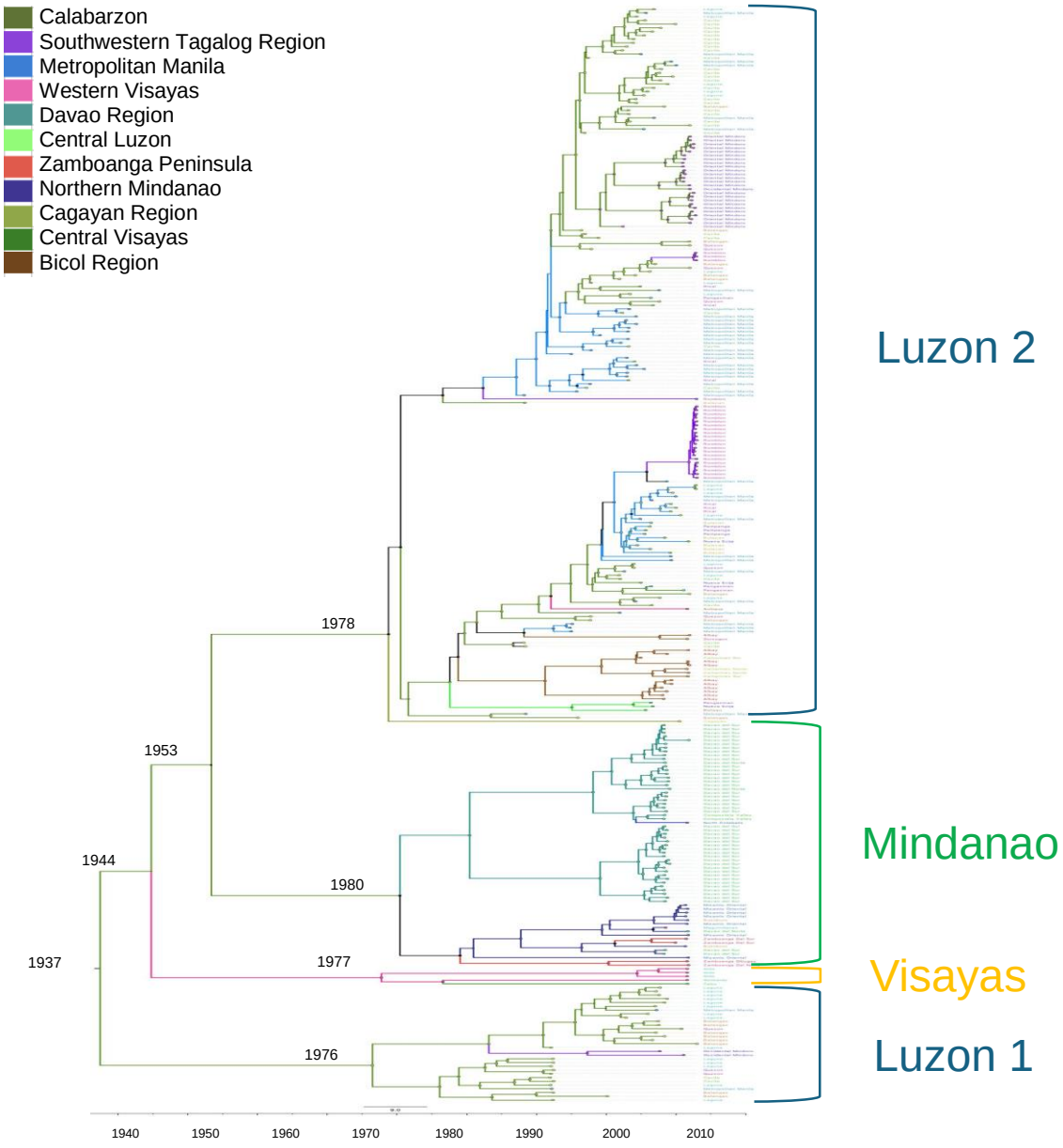

Figure 4.10**. Maximum Clade Credibility (MCC) time-scaled tree generated using BEAST, showing the phylogenetic relationships and divergence times of RABV sequences from the Philippines.** The tree reveals distinct clustering by island group, with separate monophyletic clusters corresponding to Luzon, Visayas, and Mindanao with two distinct clusters observed Luzon, suggesting multiple introductions or localized transmission chains. The time axis reflects the evolutionary timeline from 1930-2030.

4.5 Discussion

This study aimed to explore the temporal patterns of genetic diversity and geographic distribution of RABVs at a finer scale than previous studies, while also elucidating more on the evolutionary history and contemporary distribution of RABVs in the Philippines. Using phylogenetic methods, the results reveal strong geographic associations that can be leveraged to target rabies control efforts more effectively, focusing resources on specific regions to interrupt transmission pathways.

RABV sequencing in the Philippines has been ongoing since early 1990s, with most studies focusing on partial G gene sequences generated over a decade ago. However, viral evolution and transmission patterns may have since changed. This study leverages WGS to provide a more detailed and contemporary understanding of RABV transmission. While partial genome sequencing offers valuable insights WGS enhances resolution for tracing transmission pathways (Dudas et al., 2017), identifying emerging variants (Lu et al., 2020) and is key to understanding viral evolution (Pybus & Rambaut, 2009), pathogenicity, and spread. This study generated the largest set of whole genome sequences for RABV in the Philippines to date (n=208), spanning a 25-year collection period from 1998 to 2023. Molecular clock analysis estimates the MRCA to 1932, indicating nearly a century of RABV circulation, with Luzon hosting the oldest lineage. Molecular clock analysis estimates the MRCA to 1032, indicating nearly a century of RABV circulation, with Luzon hosting the oldest lineage. This aligns with previous findings by Tohma et al., which identified Region IV in Luzon as the origin of the Philippine MRCA. The same study, which used partial G gene sequences estimated the MRCA of the Asian 2b (where the Philippine RABV lineages diverged) to have emerged later, around 1967, following its divergence from the Chinese Asian 2a lineage (Tohma et al., 2014). In this study, MCC trees, generated from partial N and G genes produced comparable clade structures but demonstrated reduced temporal resolution compared to whole genome sequences. This indicates greater uncertainty in node dating and divergence times, likely due to the limited phylogenetic signal present in shorter genomic regions and added value of whole genome data in evolutionary analyses and temporal reconstruction.

The subsequent emergence of RABV in Visayas, followed by Mindanao, suggests a north-to-south pattern of spread as RABV dispersed throughout the country. Emergence in Mindanao was estimated to have occurred around 1980, indicating a relatively recent

introduction and circulation on this island. The geographic isolation of Mindanao from the rest of the Philippines, with access to the island primarily through air and sea transport and fewer ports and airports compared to more densely populated Luzon, likely slowed the virus's dispersal to the island from the rest of the country. Mindanao also has conflict zones that could be acting as a barrier to rabies introduction, limiting internal movement within and reducing the likelihood of rabies being introduced to these regions from outside the island.

The increase in RABV diversity in the Philippines observed around mid-1980's and subsequent fluctuations after 2010 is suggestive of an increased spread of RABV in the last 40 years, including the emergence of new lineages. A significant loss of genetic diversity was observed from 2022 onwards, despite an increase in genetic data available during this period (as indicated by narrowing of uncertainty over time). This suggests a population bottleneck or other event- for example, disease control interventions- that has reduced the genetic diversity in the population recently. However, this could also reflect biases in sample selection given the focus on the outbreak investigation in Romblon Province (**Error! Reference source not found.**). Identifying factors leading to this decline could help in designing targeted interventions. However, I did note problems with the Bayesian skyline model in my analysis that will need to be addressed before drawing any solid conclusions, therefore this result should be interpreted with caution. In particular, several key parameters, including those related to population size changes had low ESS despite long MCMC chain lengths. This suggests poor mixing or convergence issues, which can arise from overly complex model settings, insufficient signal in the data to estimate population dynamics, or limited temporal structure in the dataset.

Phylogenetic analysis revealed four major island-based clades, corresponding to the geographic island groups of Luzon, Visayas, and Mindanao, with two distinct clusters in Luzon confirming previous studies (Saito et al., 2013; Tohma et al., 2014). The strong phylogeographic structure observed among the islands, along with minimal evidence of inter-island spread, supports the implementation of island-specific control programs. For example, the Visayas, being a cluster of smaller, discontinuous islands, offer easier border control since inter-island entry and exit points are clearly defined, making it simpler to enforce regulations. In contrast, Luzon's shared land borders allow for more frequent, often unmonitored, translocation within the island through land transport. A control strategy for Luzon should focus on regional collaboration, prioritizing vaccination campaigns along the borders and working inward toward the central areas. Additionally, in island-specific rabies

control, detecting and monitoring incursions is crucial, as a single outbreak in a previously rabies-free area, like the Romblon incident, can undermine years of progress (Chapter 5).

Spatial reconstruction revealed a clear north-to-south dispersal pattern, suggesting that RABV was likely introduced from Luzon, primarily through human-mediated movement. Luzon appears to have served as the origin of early RABV introductions, with subsequent spread to the Visayas and Mindanao, potentially facilitated by unregulated movement of dogs, through trade or relocation. The persistence of localised transmissions within island groups, with occasional long-distance dispersal events, highlights the need to reinforce inter-island movement controls and enhance surveillance efforts to prevent further geographic expansion of RABV.

While significant phylogeographic structure was also observed at regional and provincial levels, this structure appeared more eroded at finer resolutions, revealing a greater number of co-circulating lineages and instances of inter-regional and inter-provincial admixture. Therefore, within the major island groups cross-regional collaboration, particularly between regions with closely related viral lineages, will be required to ensure synchronized control efforts like coordinated mass vaccinations and outbreak response plans. This appears to be particularly relevant in southern Luzon regions, including Metro Manila, where the greatest lineage diversity was observed. Although this pattern may be partly explained by sampling bias, it also reflects Luzon's role as a central transportation hub, facilitating viral transmission across regions. The inter-regional viral movement and prevalent local transmission indicate that dog rabies is not being effectively controlled in many provinces. This spread, likely driven by human-mediated dog movements, highlights the need for region-specific strategies. For example, stricter quarantine and animal movement regulations, enhanced monitoring, and increased dog vaccination in central transportation hubs and high-traffic areas like Metro Manila may also help to limit the spread of the virus. People frequently move between urban and rural areas, often bringing pets, particularly dogs, with them. This regular movement increases the risk of spreading distinct viral lineages from one region to another, especially if these animals are not properly vaccinated. Areas with distinct viral lineages may have unique transmission dynamics, making it essential to tailor control measures to ensure interventions are suited to local conditions. For example, Calabarzon shows a high diversity of RABV lineages, indicating that it is a high-risk zone that may benefit from improved rabies control efforts. Localized public awareness campaigns can inform the community and help authorities to focus on local transmission risks or emphasize the importance of reporting rabies cases in identified hotspots. Similar to

the efforts in Romblon (see Chapter 5), training public health and animal sector workers in sample collection and reporting can strengthen surveillance and enhance rabies control. This approach significantly improves containment and prevention efforts.

One key limitation of this study is the overall sampling biases present in the data, where some areas are represented much more extensively than others, e.g. Luzon represents 76% of WGS, Visayas only 2%. The lack of representation in certain areas poses challenges for effective surveillance, such as the potential to miss key lineages or important evolutionary changes and the ability to infer the source of RABV cases. This issue also affects phylogenetic inference, as demonstrated by the low posterior support for the phylogenetic placement of the Visayas-associated clade, which only contains 6 sequences. Despite the need for more samples to improve certainty, BaTS analysis - which also included partial sequences- clearly showed a strong geographic clustering of sequences from Visayas, suggesting that additional data would enhance the posterior support for this clade in the WGS tree. However, overall sampling bias presents an issue for more advanced phylogeographic analyses, where ancestral state reconstruction can be heavily influenced by location bias but can be addressed through representative sub-sampling and testing the robustness of results (Layan et al., 2023)

This chapter provides a contemporary overview of RABV in the Philippines, demonstrating the critical role of whole genome sequencing to improve the accuracy of inferred RABV evolutionary patterns in the Philippines and its ability to elucidate fine-scale lineage dynamics. This extensive dataset of WGS enhances genomic surveillance across the nation, strengthening rabies control programs by providing more comprehensive data. This included increasing sequencing efforts in underrepresented regions, although there are still sampling biases as discussed above, to identify emerging transmission patterns and develop region-specific strategies. Expanding surveillance is crucial for understanding viral evolution and addressing the impact of sampling limitations, particularly in underrepresented regions like the Visayas. By using real-time data from genomic sequencing, authorities can gain insights into viral migration and local transmission, enabling targeted interventions to effectively tackle rabies spread in high-risk areas. Future work will include phylogeographic analysis, to provide deeper insights into viral spread and population structure in the Philippines, including quantifying transmission between regions and correlating diffusion dynamics with ecological and epidemiological features (Brunker et al., 2018). Ultimately, there is much to exploit in this genetic data that will provide a greater understanding of the complex RABV

transmission dynamics in the Philippines that should inform more effective strategies for disease control and prevention.

4.6 Conclusion

The primary aim of this study was to analyse the evolutionary relationships and divergence patterns of RABV in the Philippines using whole genome sequencing and phylogenetic methods. The findings reveal the existence of four major clades, with the Luzon clade exhibiting the greatest genetic diversity and divergence times estimated as far back as 1932. The results highlight the long-established presence of RABV in Luzon while sub clustering within the Mindanao clade suggests local and sustained transmission post-introduction. The identification of strong geographic associations is a key finding that can inform how to implement effective control- for example, island-specific programs with cross-regional collaboration to control and manage outbreaks. The study's main contributions include the generation of the largest dataset of whole genome sequences for RABV in the Philippines, which provides greater phylogenetic resolution, and a clearer picture of transmission patterns that is valuable in formulating strategies for regional rabies control efforts. However, limited sampling in certain regions, such as the Visayas, remains a challenge for fully resolving phylogenetic relationships. Increasing genomic sampling and expanding surveillance efforts in under representative areas is crucial for a more comprehensive data-driven public health strategies for rabies control and elimination. Despite these limitations, this study significantly advances our understanding of RABV dynamics in the Philippines and serves as a critical foundation for future genomic research and public health strategies.

Chapter 5

Combining genomics and epidemiology to investigate a zoonotic outbreak: rabies in Romblon Province, Philippines

5.1 Abstract

Rabies is a viral zoonosis that kills thousands of people annually in low/middle-income countries across Africa and Asia where domestic dogs are the reservoir. ‘Zero by 30’, the global strategy to end dog-mediated human rabies, promotes a One Health approach underpinned by mass dog vaccination, post-exposure vaccination of bite victims, robust surveillance and community engagement. Using Integrated Bite Case Management (IBCM) and whole genome sequencing (WGS), we enhanced rabies surveillance to detect an outbreak in a formerly rabies-free island province in the Philippines. We inferred that the outbreak was seeded by at least three independent introductions that were identified as coming from nearby rabies-endemic provinces. Considerable transmission went undetected, and two human deaths occurred within 6 months of outbreak detection. We conclude that suspension of routine dog vaccination due to COVID-19 restrictions facilitated rabies spread from these introductions. Emergency response, consisting of awareness measures and ring vaccination, were performed, but swifter and more widespread implementation is needed to contain and eliminate the outbreak and to secure rabies freedom. Strengthened surveillance making use of new tools such as IBCM, WGS and rapid diagnostic tests can support One Health in action and progress towards the ‘Zero by 30’ goal.

5.2 Introduction

Neglected tropical diseases persist in low- and middle-income countries (LMICs), causing major economic losses, illness and death (WHO, 2021a). Treatment and elimination prospects are limited by the inequitable allocation of financial resources, resulting in high morbidities affecting over 1 billion people worldwide (WHO, 2025). Economical strategies consisting of case-finding based on observed signs and history-taking, have been used for the epidemiological investigation of outbreaks of neglected tropical diseases including dengue fever (Wang et al., 2016) and leprosy (de Sousa et al., 2020). Genomic surveillance has also proven valuable for tackling zoonotic disease emergence, including its application to outbreaks of Ebola (Quick et al., 2016), Lassa fever (Kafetzopoulou et al., 2019), Influenza (Rambo-Martin et al., 2020) and Mpox (Isidro et al., 2022), providing insights into transmission dynamics and the impacts of interventions, and therefore informing more targeted control and prevention.

Rabies is an example of a neglected zoonotic disease caused by the rabies virus (RABV). It has long been a significant public health issue, and although the disease has been eliminated

from several regions over the last century, rabies still kills thousands of people annually in Africa and Asia (Meslin & Briggs, 2013), where free-roaming dogs are common (Hampson et al., 2015). Most of the cases in these Low-and-Middle-Income Countries (LMICs) are dog-mediated. Despite being preventable through dog vaccination, rabies is re-emerging across much of Southeast Asia, including in Malaysia (Jeon et al., 2019), Indonesia (Putra et al., 2013) and Vietnam (Ward & Brookes, 2021). The historically rabies-free Timor-Leste reported its first human death due to rabies in early 2024, highlighting the ongoing challenge of spread across the region (Mali et al., 2024). Effective control of zoonoses like rabies which is transmitted between animals and humans requires a One Health approach with coordination between human and animal health sectors (Conrad et al., 2013).

Rabies is fatal once symptoms appear, but progression to disease can be prevented if immediate post-exposure prophylaxis (PEP) is given to bite victims after exposure. PEP, while highly effective, should be part of a broader rabies management strategy which includes educational campaigns to increase awareness, robust surveillance for case detection, and mass dog vaccination to interrupt dog-to-dog transmission, thereby reducing the risk of human exposures (WHO/FAO/OIE/GARC, 2018). Dog vaccination campaigns in the 20th century (Rupprecht, 2002) led to the elimination of dog-mediated rabies in North America, Western Europe, and parts of Asia, and dramatically reduced cases across Latin America (Wallace et al., 2017). Similar measures have been applied at the community level in some rabies-endemic countries, leading to local rabies-free zones like Pontianak City in Indonesia (Aptriana et al., 2022) and N'Djaména in Chad (Lechenne et al., 2017). However, introductions and re-emergence of rabies through animal importations by humans (Pieracci et al., 2024) or from natural incursions across borders occur regularly worldwide (AL-Eitan et al., 2021; Lojkić et al., 2021; Lushasi et al., 2023; Tohma et al., 2016; Trewby et al., 2017; Zinsstag et al., 2017). Examples from the city of Arequipa in Peru (Castillo-Neyra et al., 2017), Sarawak in Malaysia (Jeon et al., 2019), and Mpumalanga province, South Africa (Mkhize et al., 2010), demonstrate how neglecting surveillance and dog vaccination can lead to rapid escalation from introductions in areas close to rabies-endemic zones. Inappropriate responses, such as dog culling can also exacerbate spread as seen in Indonesian islands, Flores (Windiyaningsih et al., 2004) and Bali (Putra et al., 2013), where failure to contain the epidemic led to enzootic transmission.

The hallmark of effective rabies control is strong intersectoral collaboration to reduce human mortality risks and eliminate disease from reservoir populations, which is why a One Health approach is recommended (WHO, 2018). Incorporating a One Health approach has been

shown to address common gaps in rabies surveillance, such as poor case detection in dogs (Gibson et al., 2022). Tools like rapid diagnostic tests or RDTs (Freuling et al., 2023; Mauti et al., 2020; Naïssengar et al., 2021), regular coordination between health workers from human and animal sectors through communication technologies (Mbaipago et al., 2020; Schrodt et al., 2023), Integrated Bite Case Management (IBCM) (Etheart et al., 2017; Lushasi et al., 2023; Madjadinan et al., 2022; Ross et al., 2023), and genomic sequencing (Gigante et al., 2020b; Lushasi et al., 2023; Zinsstag et al., 2017) are known to offset surveillance weaknesses, while strengthening health systems for outbreak preparedness.

IBCM is a rabies surveillance strategy that directly links public health and veterinary workers to manage animal bite incidents and prevent rabies (Lushasi et al., 2020). IBCM was first introduced in the Philippines as part of a research project under the Surveillance Integrating Phylogenetics and Epidemiology for Elimination of Disease: Evaluation of Rabies Control in the Philippines (SPEEDIER), implemented in collaboration with the Research Institute for Tropical Medicine (RITM) and the Field Epidemiology Training Program Alumni Foundation, Inc. (FETPAFI). The introduction of IBCM to Tablas in March 2020 aimed to establish a structured, real-time reporting mechanism that would strengthen coordination between animal bite treatment centers (ABTCs) and municipal agriculture offices (MAOs). Before implementing IBCM, training workshops were conducted in municipalities through SPEEDIER, involving both human and animal health sectors. The training covered IBCM's purpose, sector roles, risk assessment using a clinical algorithm and animal investigations using an investigation algorithm, event-based reporting, and data entry using the ODK mobile application for human and animal cases.

Additionally, IBCM enhances surveillance through better case detection, improves patient care through more informed administration of PEP and can support better management of limited resources (Swedberg et al., 2022). In the Philippines, PEP remains the primary method of preventing rabies in humans. The government provides free PEP at designated animal bite treatment centers (ABTCs) in public hospitals and health units, ensuring access for those who queue for treatment. However, in private animal bite clinics, PEP is available but requires payment, which may limit access for some individuals. The country uses the updated Thai Red Cross intradermal regimen.

Genomic sequencing of rabies viruses has significantly advanced our understanding of virus transmission, emergence, and persistence. It has helped identify new virus reservoirs (Kotait et al., 2019), trace sources of introductions (Mollentze et al., 2013) and pinpoint populations

that pose risks for re-emergence, facilitating targeted interventions (Gibson et al., 2022; Lushasi et al., 2023; Mahardika et al., 2014). Beyond case detection, sequencing has provided critical insights into virus dispersal dynamics, uncovering the role of geographic, ecological, and anthropogenic factors in shaping transmission pathways (Dellicour et al., 2019; Layan et al., 2021; Nahata et al., 2021).

In 2015, the WHO launched the 'Zero by 30' global strategy to eliminate dog-mediated human rabies deaths by 2030 (WHO/FAO/OIE/GARC, 2018). However, achieving successful rabies control requires overcoming challenges such as limited human resources and cross-sectoral financing. Government priorities typically favour investment in animal diseases that have economic impacts such as African Swine Fever (ASF), whilst political and economic instability with frequent changes in governance make programmes difficult to maintain (Arias-Orozco et al., 2018). As a result, another major challenge to 'Zero By 30' is sustaining rabies freedom, as outbreaks reestablish due to lack of healthcare resources and siloing among different health departments, undermining outbreak response.

Here, we report our learning from taking a One Health approach to tracking a rabies outbreak as it unfolded in a formerly rabies-free province in the Philippines. The investigation began with the initial detection of a rabid dog in 2022 on the island of Tablas, in Romblon Province (Figure **5.1**). The island had previously suffered from an incursion in 2011, and the ensuing outbreak caused 11 human fatalities (incidence of 3.48 deaths/ 100,000 persons/ year) but no human or animal cases had been reported since 2012 (Tohma et al., 2016). Our cross-sectoral and multidisciplinary investigation used IBCM to enhance rabies surveillance as advocated by 'Zero by 30'8 and deployed RDTs for early diagnosis. We further undertook whole genome sequencing of rabies viruses from the outbreak to determine its probable origins and uncover the resulting spread.

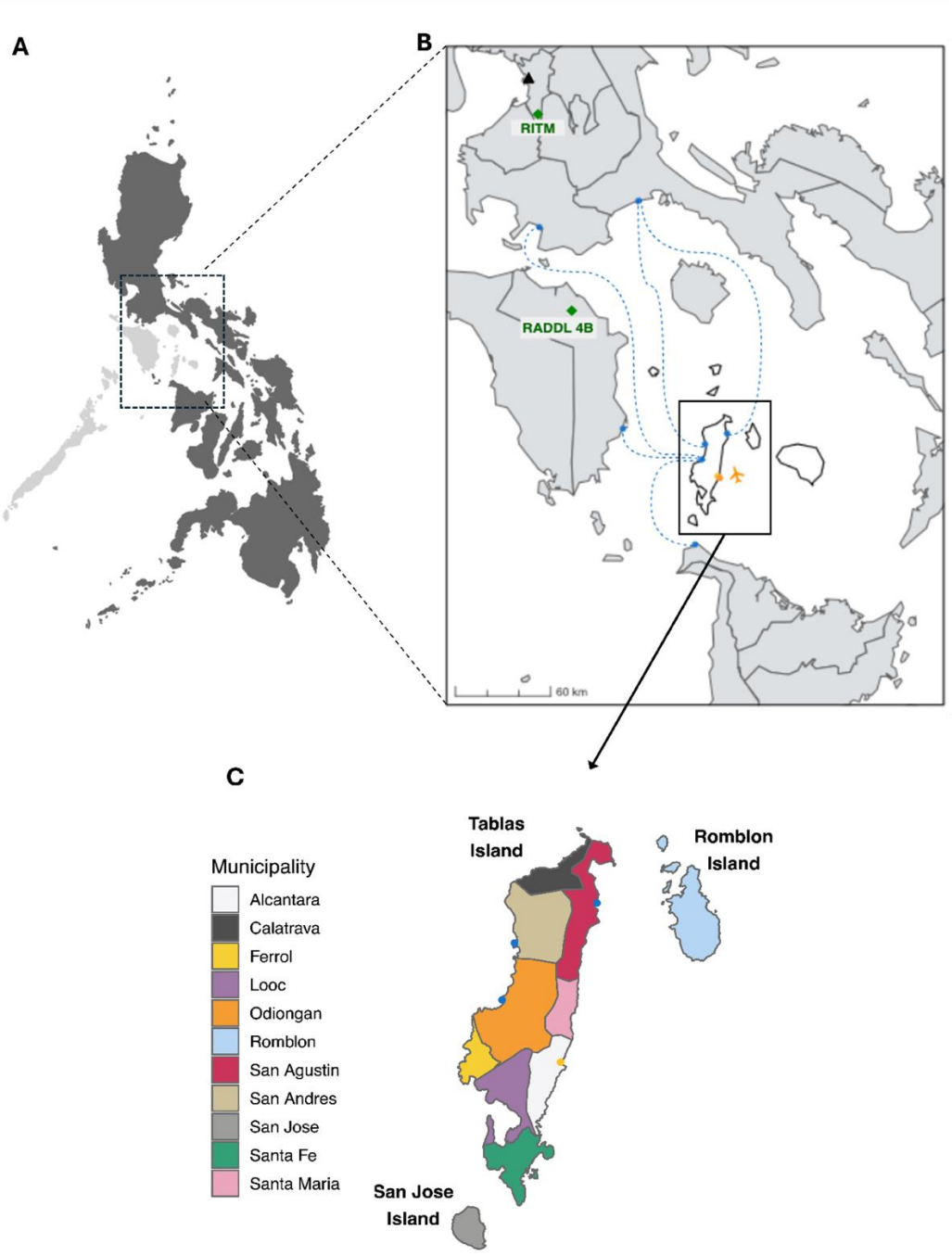

Figure 5.1**. Outbreak location in the formerly rabies-free province of Romblon in the Philippines.** A) Location of MIMAROPA region, also known as Region 4B (light grey) within the Philippines, with the inset B) of Romblon Province (white) showing the Regional Animal Disease Diagnostic Laboratory (RADDL) and the Research Institute of Tropical Medicine (RITM) as green diamonds. Manila is indicated as a black triangle. Major ports are indicated in blue, the airport in yellow and dashed blue lines show the main ferry routes to/from Tablas. C) Tablas Island coloured by municipality, with the ports and airport coloured as above.

5.3 Methods

5.3.1 Study description

This study took place on Tablas Island, Romblon Province, MIMAROPA region of the Philippines. Tablas has a human population of 174,447 (Philippine Statistics Authority, 2021) served by Odiongan, Santa Fe and Calatrava ports, and Tugdan Airport in Alcantara (Figure **5.1**). The dog population is not known, but estimates of human:dog ratios in the Philippines suggest it is between 17,445 and 58,149 (Swedberg et al., 2023). Prior to 2020, dog vaccination coverage across the province ranged from 18.0-38.6% according to regional reports and was self-reported by Romblon province from 2021 onwards as fluctuating between 0 and 24.2% (the number of vaccinated dogs per municipality can be seen in Figure D. 1.)

Ethical review was secured from RITM ethical review board (2019-023) and the University of Glasgow, Medical, Veterinary & Life Sciences ethics committee (200190123).

5.3.2 Case Finding through IBCM

Prior to IBCM, rabies surveillance in the province relied on passive reporting, where animal rabies cases were only investigated when a human exposure was reported. Confirmatory testing of suspect animals was infrequent and limited diagnostic capacity delayed case confirmation.

As part of IBCM, Public Health Workers based in animal bite treatment centres (clinics in hospitals or health units that provide PEP to bite victims) reported “high-risk” bites to animal health workers at the closest Municipal Agriculture Office (Figure **5.2**). Bites were classified as “high-risk” if the biting animal died, was killed, showed signs of poor health, was suspicious for rabies, or disappeared (Wallace et al., 2015). In animals, a case is clinically defined as any dog or other mammal exhibiting progressive neurological symptoms consistent with rabies, including aggression, excessive salivation, paralysis, or sudden death. In humans, a case was defined as any individual presenting with acute encephalitis, hydrophobia, or aerophobia following an animal bite or exposure, with no history of vaccination prior to symptom onset (WHO, 2018). A suspect case is any animal or human that is compatible with a clinical case. Probable cases are suspected cases with a reliable history of contact with a suspected or confirmed rabid animal while a confirmed case is a suspected or probable case that is confirmed in the laboratory.

If the public health workers required assistance or were busy with other duties, a Disease Surveillance Officer (appointed to coordinate IBCM across the provinces), would investigate on their behalf. Data on bite cases were systematically recorded using the ODK Collect App to ensure standardized and accurate recording. This app captured details such as the biting animal's health status, vaccination history (if known), the outcome (alive, dead, or disappeared), and the severity of the bite. High-risk cases were flagged for immediate notification to animal health workers, ensuring timely investigation and intervention. The IBCM peer support chat facilitated real-time identification of suspect rabid dogs based on unusual behaviour, even if they hadn't bitten anyone.

To facilitate case linkage, human bite cases and animal investigations were integrated into a centralized reporting system. Public health workers at ABTCs documented patient details, including clinical symptoms (if present) and exposure category, while animal health workers recorded findings from their field investigations. Reports were cross-checked and linked whenever a suspected rabid animal was identified, allowing a coordinated response. This linkage aimed to ensure that humans potentially exposed to rabid animals were prioritized for appropriate medical and epidemiological follow-up.

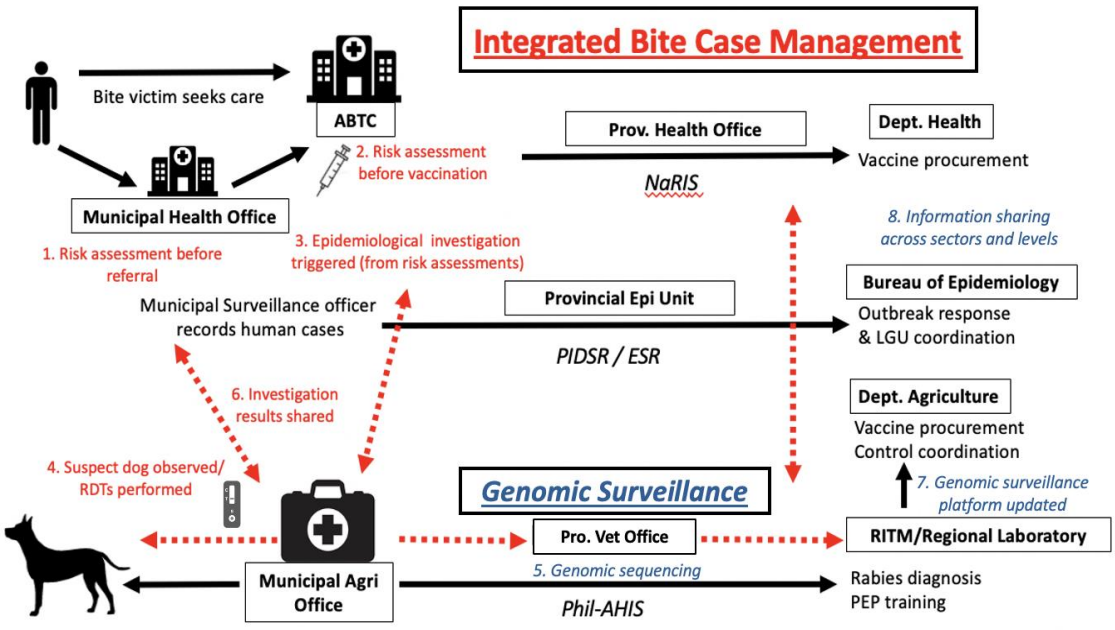

Figure 5.2**. Diagram of the complete integrated bite case management (IBCM) process.** Activities that enhance surveillance through IBCM shown in red, while genomic surveillance activities are shown in blue. ABTC = Animal Bite Treatment Center, LGU = Local Government Unit, NaRIS = National Rabies Information System, Phil-AHIS = Philippine Animal Health Information System, PEP = Post-exposure prophylaxis, PIDSR/ESR = Philippines Integrated Disease Surveillance and Response/Event-based Surveillance and Response, RDT = Rapid diagnostic test, RITM = Research Institute for Tropical Medicine

Animal investigations were initiated by phone or in-person to gather case details. Animal health workers investigated suspicious animals to confirm their health status. In the investigation process, if the animals needed confinement for observation to prevent transmission, it was made sure that the procedure was properly explained to the owner and follow up of the animal status while in confinement was carried out. Sick animals or animals presenting signs of rabies were quarantined at the owner's house, for a 10-day observation period (WHO, 2013), as formal quarantine facilities were often unavailable. During this process, animal health workers ensured that the rationale and procedures for home confinement were clearly explained to owners, including information about the risks of rabies and the importance of monitoring. Follow up visits or communications were conducted to track the animals' condition throughout the quarantine period and to support early detection and response should clinical signs appear.

Dead animals were subject to sample collection. Through IBCM, the use of RDTs was first introduced to enhance early rabies detection and response. The Bionote RDT (Bionote, 2024), a lateral flow immunoassay that detects rabies virus antigens in brain tissue, was deployed in the field by trained animal health workers. This provided a rapid, on-site preliminary diagnosis without the need for specialized laboratory equipment. If available, brain tissue, the head or the whole carcass was collected, and testing was conducted immediately. While RDT offered quick results to guide immediate action, additional samples were packaged and sent for laboratory confirmation to ensure diagnostic accuracy. If sample collection did not coincide with scheduled ferry trips, samples were temporarily stored frozen in the Provincial Veterinary Office, then sent to RADDL 4B for DFAT.

As ferry trips were normally scheduled on weekends and outside office hours, one Provincial Veterinary Office staff delivered samples in batches, depending on their availability. When confirmatory testing was not possible at RADDL (fluorescent microscope was broken), samples were instead submitted to the RITM National Reference Laboratory in Manila (Figure **5.1**). Human samples (saliva and or nuchal skin biopsy) from suspected cases, whether pre- or post-mortem, were collected and sent to RITM for confirmation through nested PCR (Figure **5.2**) (Dacheux & Bourhy, 2018). As part of routine procedures when handling a probable rabies patient, hospitals sought informed consent before conducting sample collection, with a statement clarifying that laboratory results were to be performed for surveillance purposes. Samples that were confirmed positive by DFAT or PCR were stored at -80°C at the laboratory for banking and research purposes.

5.3.3 Whole genome sequencing and phylogenetic analysis

Twenty-four out of 43 rabies-positive samples from the outbreak were sequenced (Table D. 1), following a previously established protocol for whole-genome sequencing of RABV (Bautista et al., 2023) to maximise reagent use, periodic sequencing was conducted, with 12-23 samples per run.

A Romblon-only phylogenetic tree using whole genome sequences was generated in IQtree and Romblon sequences were divided into phylogenetic lineages, for transmission tree inference (see next section). Lineages were defined through patristic distance clustering with the adegenet package (Jombart, 2008), using a threshold of 0.0004, determined by comparing patristic distance clusters with phylogenetic trees and considering the RABV evolutionary rate (~2x10-4 substitutions/site/year). A heatmap of patristic distances is available in Figure D. 3

A large contextual dataset of partial and whole genome Philippine RABV sequences (n=615) from the RABV-GLUE database (n=694) (Campbell et al., 2022) was obtained for this study, with additional recently published genomes (Bacus et al., 2021) (n=49), and whole genome sequences (WGS) from an ongoing Philippine RABV study (n=4) and this outbreak (n=24). The contextual data constituted RABV sequences with the Philippines as the country of origin, which belong exclusively to the Asian SEA4 clade, a phylogenetic clade associated with and almost entirely restricted to the Philippines (we did not include 11 sequences found in other countries). Associated metadata was prepared using custom R scripts to clean and standardise data from the different sources, including merging of partial sequences with the same sample IDs (but submitted under different GenBank accession IDs). Overall, this resulted in a dataset of 581 sequences. Metadata, sequences and code can be found in the GitHub repository: https://github.com/boydorr/outbreak_romblon_PHL. To prepare a sequence alignment, concatenated WGS (n=79) were aligned using MAFFT (v7.520) (Katoh & Standley, 2013) with default parameters, then added to the RABV-GLUE downloaded alignment using MAFFT's functionality to add full length sequences to an existing multiple sequence alignment with the keeplength option on. Each alignment was checked and edited manually as required (minor edits).

Phylogenetic analysis including tree dating and ancestral character reconstruction was performed following the methods in Holtz et al (Holtz et al., 2023). A maximum likelihood tree was constructed from the 581 Philippines RABV sequence data using FastTree v2.1.11

(Price et al., 2010) with a GTR+Gamma20 model. Using the sequence-associated dates, this tree was rooted according to the best root-to-tip correlation, by running the initRoot function in R package BactDating (https://github.com/xavierdidelot/BactDating). The rooted tree was pruned to WGS only using gotree (v0.4.5) and the evolutionary rate estimated using the R-wrapper for lsd2 (To et al., 2016) (Rlsd2) with a ZscoreOutlier of 3. This rate estimate was used as a prior to inform tree dating for the full 581 sequence RABV tree with a ZscoreOutlier of 5 and 1000 bootstraps to generate date CIs. PastML (Ishikawa et al., 2019) (v1.9.43) was used to perform ancestral character reconstructions on the dated tree using a marginal posterior probabilities approximation, with Philippines administrative divisions as traits (region and province). Subtrees including recent Romblon outbreak sequences and their 10 closest relatives were extracted from the larger contextual phylogeny for interpretation. Romblon phylogenetic lineages were defined according to a patristic distance threshold of 0.0004. Tree annotation and visualisation was performed in R with the ggtree package (Yu et al., 2017).

Outbreak spread between introduction (T_{int}) and first detection (T_{obs}) was estimated using a branching process model, simulating the serial intervals and secondary cases probabilistically from lognormal (meanlog = 2.85, sdlog = 0.966) and negative binomial distributions (mean = 1.20, k = 1.33), respectively, to generate descendants from the initial case (Mancy et al., 2022; Townsend et al., 2013b). The interval between T_{int}, inferred via phylogenetic analysis, and T_{obs} was calculated to determine simulation run time, conditioned on outbreak persistence until T_{obs}. 1000 outbreaks were simulated, and the median and 95% prediction intervals of undetected cases calculated. As this model assumes an infinite susceptible population, the median and prediction interval were calculated only from plausible outbreaks (incidence not exceeding 1% of Romblon's dog population).

5.3.4 Transmission tree inference

We probabilistically reconstructed transmission trees using the treerabid R package that generates trees consistent with phylogenies (Rajeev & Bogaardt, 2024). Progenitors for each case were inferred from reference distributions of the rabies dispersal kernel and serial interval (Lognormal serial interval, meanlog 2.85, sdlog 0.966 and Weibull distance kernel, shape 0.698, scale 1263.461) (Mancy et al., 2022). We incorporated uncertainties into our bootstrapping procedure for dates of case onset and case locations, since the barangay was recorded for each case, but geolocations were not. Specifically, for each bootstrap, we assigned case onset dates by sampling uniformly from a 5-day window up to and including

the date of the biting incident or sampling if this was reported, or a 15-day window up to and including the date of laboratory submission or testing otherwise. We selected plausible case localities by sampling from 100x100m raster grid cells in proportion to population density according to unconstrained model data from worldpop (WorldPop, 2018).

We generated 1000 bootstrapped trees for each of 32 scenarios, corresponding to all combinations of the following: (i) case locations (barangay centroids versus locations sampled from the population density grid); (ii) use of genetic data for inference (yes/no); and (iii) inclusion of pruning steps to further resolve transmission chains (eight different combinations of cut-offs). In the scenarios using genetic data, transmission trees were first constructed using spatiotemporal data as described above and then made consistent with phylogenetic lineage assignments by using a rewiring algorithm for cases assigned to incongruent lineages. In the phylogenetic lineage assignments, we interpolated the existence of an unsampled rabid dog at the time and location of the human exposure that developed rabies and for which a sequence was obtained, and assumed this case belonged to the designated lineage. In the scenarios with additional pruning steps, case pairs were filtered out where the time interval and/or distance exceeded specified percentiles of the serial interval and distance kernel distributions. Pruning options included no pruning, pruning by time only (cut-offs 0.95, 0.975 and 0.99), pruning by time and distance using the same cut-offs (0.95, 0.975 and 0.99) and one combination of differing cut-offs (time 0.975, distance 0.99). Without pruning or integration of phylogenetic information, tree reconstruction results in a single chain. The different scenarios were compared on the basis of their consensus trees.

5.4 Results

5.4.1 Rabies cases

Romblon province was considered rabies-free, with no cases recorded since 2012, until 2020 when two suspicious human deaths occurred in Romblon Island (Figure **5.3**A). Prior to 2012, four human deaths were recorded in the province between 2003-2006. An outbreak on Tablas island in 2011 confirmed eight animal cases and 11 human deaths (Tohma et al., 2016). From 2017 to 2019, animal sample submissions ranged from 6 to 39 annually, but dropped to <5 during lockdown. In late 2022, the use of IBCM identified a cluster of bite cases leading to the detection of the first dog rabies cases on Tablas Island, Romblon Province, in over a decade.

The first detected rabies-positive case (November 21st, 2022) was a dog that was investigated three days after its involvement in a biting incident (November 18th) in Santa Maria municipality. This was the first sample from the province to have been tested for rabies since 2020, and the first local use of an RDT after being supplied for IBCM (training carried out in March 2020 just before COVID-19 restrictions were announced). Due to the absence of laboratory facilities in Romblon province and the fluorescent microscope being broken at the RADDL 4B, (Figure **5.1**), the sample was transported overnight to the National Reference Laboratory at the RITM in Manila. Here it was confirmed the next day (November 22nd) through direct fluorescent antibody testing (DFAT) and the positive result was immediately communicated to the local government, prompting increased sample collection and in-field testing. That week two more samples collected from biting dogs in Odiongan and Alcantara municipalities were sent to RADDL 4B where they tested positive by RDT. Another biting dog from San Agustin municipality was classified as probable rabies after being killed and consumed without sample collection. Positive confirmation of the first case in Santa Maria municipality prompted the sending of two frozen dog heads collected from Alcantara municipality in September and October 2022 to RITM in early December 2022. Both tested positive via DFAT, thus marking the index case of the outbreak as September 30th, 2022).

Between September 2022 and September 2023, a total of 43 animal rabies cases and two human deaths were confirmed in eight out of the nine municipalities in Tablas Island (Figure **5.3**C). Additionally, three biting dogs were classified as probable cases, based on clinical signs and progressive fatal outcomes consistent with those reported in literature (Ma et al., 2020; Medley et al., 2017), but without diagnostic confirmation due to lack of sample collection. The One Health link between public health and veterinary workers operationalized through IBCM was critical to identifying many of the rabid dogs (n = 25). Conversations on the IBCM peer support chat also disseminated information about rabid dogs that were investigated directly because of their strange behaviour (i.e. not because of biting a person). The Disease Surveillance Officer facilitated resource sharing between sectors by transporting supplies, case reports and vehicles so that investigations were conducted within 1-2 days of an animal death, before samples decomposed or became unfit for testing.

Of the submitted samples, 71.7% (43/60) tested positive by DFAT (all dogs). Only 3.3% (2/60) of submitted samples were from cats and both were negative. RDTs were used for initial screening of 51.7% (31/60) of samples. All RDT-positive samples were confirmed by

DFAT. The RDT specificity was 100%, while sensitivity was 95.7%, with one initially negative sample later confirmed positive by DFAT. Most positive cases were detected in San Agustin municipality (n=13/43, 30.2%) where one of Tablas' ports is located (Figure **5.1**). San Agustin had the highest sample submission rate, followed by Odiongan municipality, which accounted for 25.6% (11/43) of positive cases. No samples were collected from Ferrol municipality, nor were any probable rabid animals reported there. Most rabies-positive dogs were owned, while no owner could be identified for 32.6% (14/43). Twenty three point five percent (4/17) of rabies-negative dogs had a history of vaccination, while 13.6% (6/43) of rabies-positive dogs had reportedly been vaccinated, although the vaccination year was unspecified, except for one of the dogs that became ill and died shortly after vaccination in 2023. Rabies-positive animals were either killed (27.9%), found dead (23.3%), died while under observation (41.9%) or had unspecified outcomes (7%).

Two human rabies deaths were identified in 2023: one in February (39 days after the bite in December 2022) and another in May (131 days after the bite). The victims, a child from Santa Maria municipality and an elderly person from Odiongan municipality, were bitten by dogs and did not receive PEP. Initially, they sought treatment from local faith healers ('tandok'), as encouraged by their families and were hospitalised only when symptoms worsened.

On average, the delay between exposure and PEP in treated patients was 1.8 days (95% CI: 0.14 - 3.36 days; median of 0 days; n=12). The mean delay between a biting incident and dog death in confirmed cases was 2.1 days (95% Confidence Interval (CI): 0.7 - 3.5 days), and a median of 1 day.

5.4.2 Rabies control and prevention

Since 2000, rabies control in Romblon Province has primarily involved yearly mass dog vaccinations, with estimated coverage never exceeding 40%. During the COVID-19 pandemic, dog vaccination campaigns were suspended due to social distancing restrictions and resource reallocation leading to a decline in coverage (Figure **5.3**A). Air travel to and from Tablas was suspended from March 2020 to December 2022, but inter-island ferries continued routes via rabies-endemic provinces of Oriental Mindoro, Quezon and Batangas to ports in Odiongan, San Agustin and Calatrava municipalities. While pets are allowed on ferries with a health certificate and proof of rabies vaccination, in practice, checks at public

ports are rare. Additionally, private pump boats frequently used by fishermen, tourists and visiting families do not subject companion animals to regulatory procedures.

The confirmation of the positive animal rabies case in Santa Maria municipality in November 2022 prompted an immediate state of emergency declaration by the municipal mayor (Figure **5.3**B). Contact tracing identified humans and animals exposed to the rabid dog, and ring vaccination of 66 dogs within the village was conducted. However, municipality-wide dog vaccination was not carried out due to limited human resources and vaccines. In March 2023, after the first human rabies case, an 'Information, Education and Communication' (IEC) activity, consisting of lectures on rabies prevention, was held in the victim's *barangay* (village). That same month, the governor of Romblon Province instructed all municipalities' mayors on Tablas Island to enforce Republic Act No. 9482 (Anti-Rabies Act of 2007), requiring local government units to allocate funds toward dog vaccination (Government of the Philippines, 2007). Subsequent dog vaccinations were both limited and heterogeneous across the island. While some municipalities restarted vaccination campaigns in 2022, at least three did not conduct large-scale dog vaccinations in 2022 or 2023 (Figure D. 1). As a result, the proportion of the dog population vaccinated declined, from 24.2% vaccinated in 2022, to just 8.2% in 2023. A regional workshop held in early 2024 has since catalysed concerted island-wide vaccination that was completed in May 2024 (12,792 dogs vaccinated).

Bite patients are generally administered PEP for free on presentation to animal bite treatment centres. However, following the release of positive DFAT results, there was an increase in the patients presenting that underwent IBCM risk assessments, with bite victims duly encouraged to complete PEP regimens. Additional contact tracing was conducted but no other bite victims were identified. No human fatalities were attributed to laboratory-confirmed rabies cases. Delays in DFAT results with an average of 6 days (2-10 days) were circumvented, with RDT results used to reinforce tracing of bite victims. However, lack of recognition of RDTs at national level was seen as an obstacle that prevented regional and provincial managers from declaring cases and a status based on clinical suspicion was presumed to not carry the same weight as the confirmatory test (DFAT). Contact tracing and PEP were also incomplete for some people exposed to one of the probable cases; they had consumed the dog and were hesitant to come forward, fearing repercussions since dog consumption is illegal.

Details of ongoing cases and updates to the epidemiological situation are maintained on the dashboard: https://boydorr.gla.ac.uk/rabies/SPEEDIER/

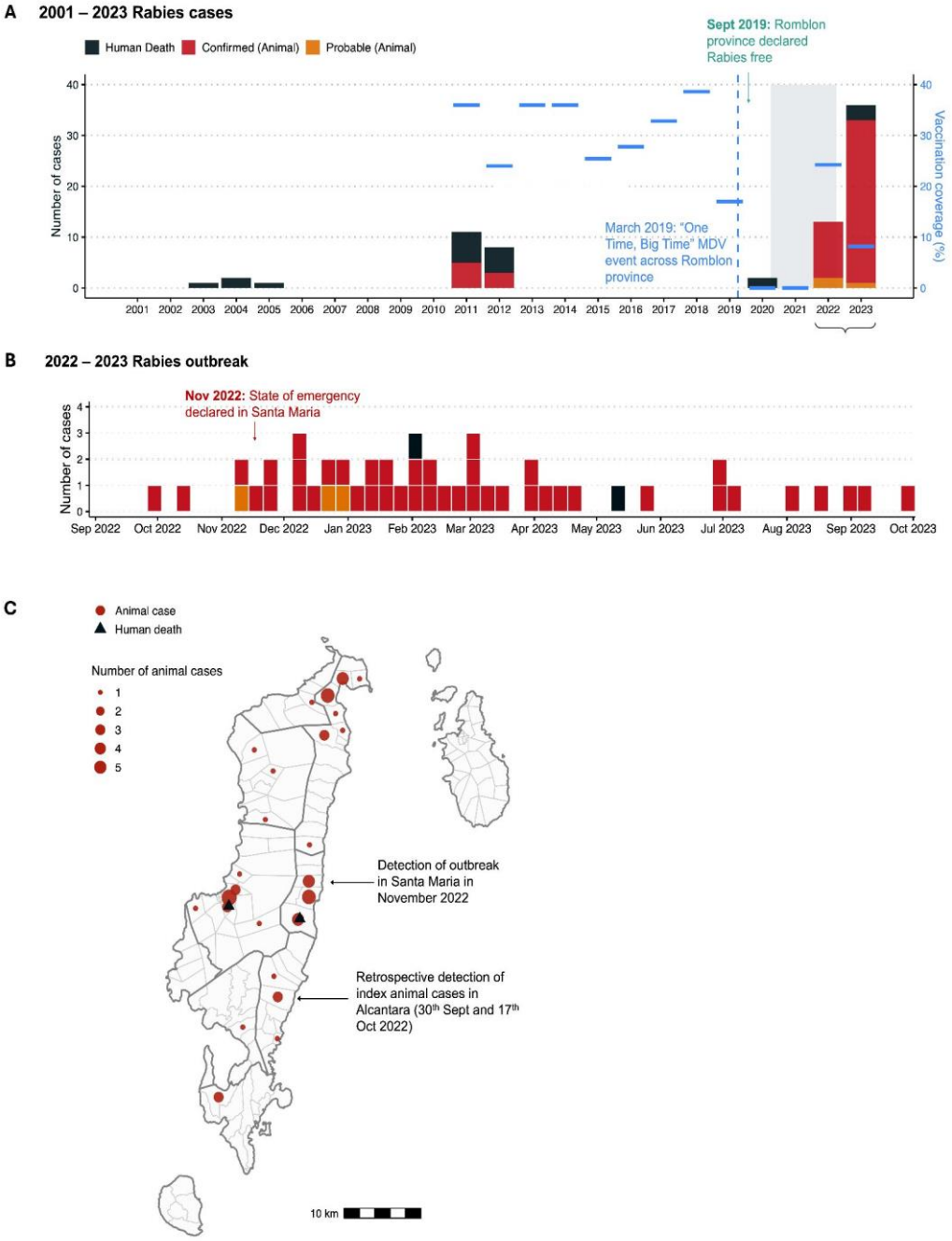

Figure 5.3**. Rabies cases and control measures recorded in Romblon province since 2001.** A.) Human deaths and confirmed and probable animal cases recorded annually between 2001 and September 2023; this includes 2 human deaths from Romblon Island in 2020 that were not confirmed so were not included in official government statistics. The estimated percentage of dogs vaccinated each year is shown as blue horizontal bars. The shaded area represents the COVID-19 lockdown period. B.) Human deaths confirmed, and probable animal cases recorded during the outbreak between September 2022 and September 2023. Human cases are dated by month of death while animal cases are dated by month of biting incident (if known); otherwise, sample collection date is used (22/43 cases). C.) Animal cases (red circles, scaled by number) and human deaths (black triangles) in Romblon between September 2022 and September 2023. Grey polygons indicate municipalities and cases detected early in the outbreak are annotated.

5.4.3 Phylogenetic inference

During the outbreak investigation, periodic genomic sequencing was carried out from December 2022 to March 2023. During this period, 96.15% (25/26) of the confirmed positive samples were successfully sequenced. One sample had insufficient genome coverage for analysis. Among the sequenced samples, 23/25 DFAT-confirmed animal brain samples achieved genome coverage of 90-99% while the human skin biopsy sample, confirmed by nested PCR, had a lower coverage of 88%.

The first sequencing run in December included the first three positive samples from November 2022. The second run on March 2nd, 2023, sequenced the remaining cases from 2022 (including the two earlier cases from Alcantara municipality since sent to RITM), and the first human case. The third run on March 8th, 2023 (six months after the index case was collected), included the remaining 14 samples up to the most recent at that time (March 1st, 2023) although three prior samples were subsequently traced to the Provincial Veterinary Office. Each sequencing run processed 12-23 samples, including additional contextual samples from other parts of the Philippines. Results from all sequencing runs were disseminated within three months, with initial findings shared in January 2023 to relevant stakeholders to inform outbreak response and control measures. A regional meeting, facilitated through SPEEDIER, brought together provincial veterinarians, animal health workers (from provinces within the region) and the RADDL, where initial phylogenetic analysis was presented, explaining that the outbreak sequences were most closely related to those from nearby provinces. This dissemination has supported plans between provincial veterinarians to do another One Time Big Time vaccination drive within the region (which is a coordinated effort between stakeholders aiming to vaccinate dogs across the region), a campaign they have done in the past prior to COVID pandemic.

Further in-depth analysis of the sequences showed the following results. A maximum likelihood tree constructed from publicly available sequences from the Philippines (n=664, reducing to 553 after excluding duplicates and consolidating genes from the same sample) plus 28 sequences from this study (24 outbreak cases, 4 isolated from nearby provinces) provided temporal and geographic context for the outbreak sequences. These 581 unique sequences were collected from 1998 to 2023 from different regions in the country and were of varying length (211 to 11,797 bp), covering different regions of the genome (further details provided in the Github repository, see methods) (https://github.com/boydorr/outbreak_romblon_PHL). Both time-scaled and substitution-

scaled trees for these 581 sequences are shown in Figure D. 2. Examining the sequences from the current outbreak within the larger contextual phylogeny revealed that at least three independent introductions occurred on Tablas Island during this outbreak (Figure D. 2A). The largest cluster of cases (n=20) subdivides into three identifiable genetic lineages (1, 4 & 5) based on a patristic distance threshold of 0.0004 (Figure D. 3 shows a heatmap of patristic distances between the outbreak sequences). These lineages may have been due to either a single introduction or multiple introductions from a single focus over a short period. The second (n=1) and third clusters (n=3) represent another two introductions, each comprising single genetic lineages (2 and 3 respectively, Figure **5.4**).

The first and largest cluster shares a historical ancestor with sequences from the previous Tablas outbreak (2011-2012). However, ancestral character reconstruction (ACR) determined the earlier and current outbreaks to have emerged from different geographic sources, specifically Pangasinan and Bulacan provinces, respectively, with their time to the most recent common ancestor (tMRCA) estimated as 2010 (Figure **5.4**). This first cluster shows several polytomies, each with 'star-like' bursts, indicative of an introduction from a common source, succeeded by multiple local transmission chains. The star-like signatures signify rapid dissemination within a naive population (Volz et al., 2013), making it highly unlikely that these cases resulted from sustained cryptic circulation on Tablas island from the previous 2011 outbreak. Based on the clusters tMRCA, we estimate an introduction around July 2021 (95%CI: Jul 2020-Jun 2022), prior to divergence into three sampled genetic lineages (Figure **5.4**A). The resulting cases are most closely related to sequences from Central Luzon and National Capital Region i.e., municipalities within Metropolitan Manila, and Bulacan province is the inferred ancestral location (marginal probability of 100%). If the different lineages were from multiple introductions, they all likely arose from Bulacan province.

The second cluster comprised just one case (the sequenced human case with 88% genome coverage), which lies on a distinct outlier branch in the phylogeny (Figure **5.4**B). It was ancestral to a large cluster of sequences (n=167) from a mixture of geographic regions, including cases from the third cluster (collapsed clade, Figure **5.4**B), with tMRCA estimated as Oct 1990 (95% CI: Sep 1985-Jul 1994). The geographic source that led to this human case however could not be pinpointed, likely due to undersampled diversity in this part of the phylogeny. We estimate that the third cluster resulted from an introduction in late June 2022 (95% CI: Aug 2021-Dec 2022) and was most closely related to sequences from

neighbouring provinces within the Calabarzon region, with Batangas province the inferred ancestral location (marginal probability of 97.7%) (Figure **5.4**C).

From simulating a branching process using epidemiological parameters (R0 and serial interval) for rabies viruses, we estimated possible cases resulting from each initial unobserved introduction to Tablas Island. Using the estimated introduction dates for two clusters (1 and 3), we estimated detection delays of 429 days for cluster 1 (from 28/7/2021 to 30/9/2022), and 141 days for cluster 3 (from 28/6/2022 to 14/11/2022). Simulations with realistic incidence suggest a median of 149 undetected cases (95% prediction interval (95%PI): 14 - 355) for cluster 1 and 30 (95%PI: 2 - 180) for cluster 3 before detection. However, if cluster 1 actually resulted from multiple introductions (estimated around 24/9/2022 and 26/8/2022), leading to lineages 1 and 5, we estimate detection delays of 6 and 121 days respectively, suggesting a median of 1 (95%PI: 1 - 3) and 20.5 (95%PI: 1 - 114.1) undetected cases. Since lineage 4 is represented by a singleton, we presume that it emerged after July 2021.

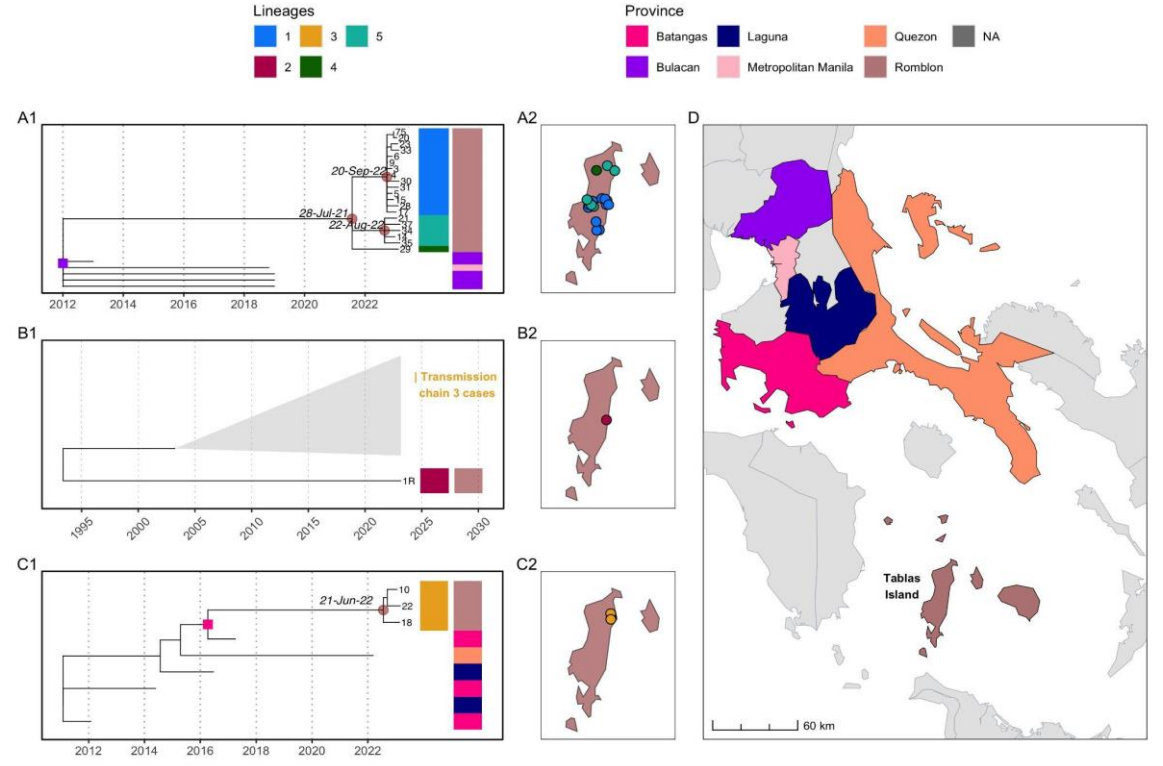

Figure 5.4. **Time-scaled phylogenetic subtrees from the current outbreak.** The source of introductions was inferred by ancestral character reconstruction (ACR). A1-C1) Subtrees corresponding to each inferred introduction, with colourstrips indicating lineages (colours match Figure 5.5) identified from clustering by patristic distance (Figure D. 3) and sequenced case locations (province-level) to match panel D. Internal nodes mark the tMRCA of each cluster and lineage (circles) and inferred province-level ACR location (squares) for each cluster's ancestral node coloured accordingly. A2-C2) Locations of sequences from Tablas (with points jittered) coloured by lineage and D) provinces in the Philippines coloured according to ACR.

Transmission trees constructed solely from epidemiological data (dates and locations) were not phylogenetically consistent, highlighting the enhanced resolution provided by viral genomes (Figure D. 4). Following rewiring for phylogenetic consistency with the 5 differentiated genetic lineages, pruning by serial interval distribution percentiles (95th, 97.5th and 99th) resulted in negligible tree configuration changes. Further pruning by distance kernel percentiles led to orphaned cases and short unsampled transmission chains, indicative of either undetected cases in areas with ongoing transmission, or of long-distance human-mediated translocations. Transmission trees inferred using *barangay* centroids versus simulated locations (in proportion to population density) were broadly similar. Using the 99th pruning percentiles split the 5 lineages into 7 transmission chains as indicated by colour in Figure **5.5**. Lineage 1 split into two transmission chains; lineage 2 linked the biting dog responsible for the first human death with an unsampled dog; lineage 3 remained as one chain and lineage 4 as a singleton; while lineage 5 split into two chains (Figure **5.5**).

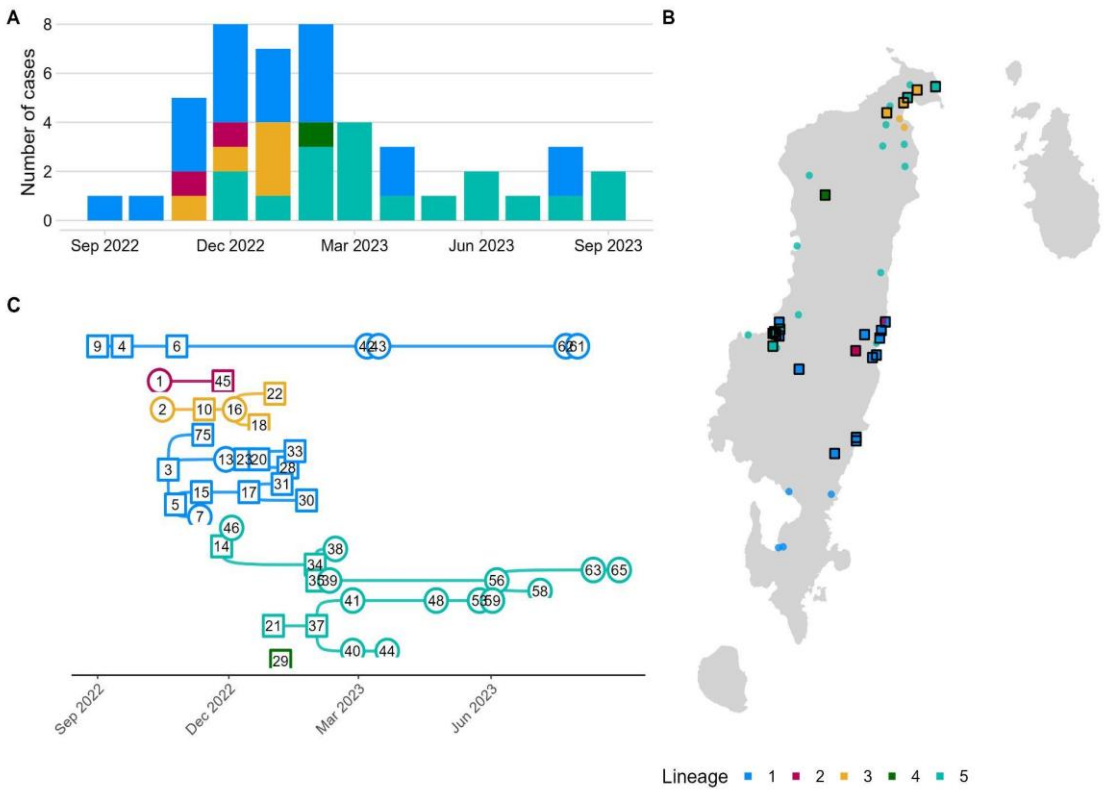

Figure 5.5**. Inferred transmission chains from the outbreak**. A.) Monthly confirmed and probable dog cases; B.) reconstructed transmission chains and C.) mapped dog case locations all coloured by lineage (as per Figure 5.4). Squares represent sequenced cases, and circles unsequenced (unsampled) cases, except for case 45, which was not sequenced, but assigned to lineage 2 based on its epidemiological link to the sequenced human case (not shown). The illustrated chains are from the consensus transmission tree with case locations simulated in proportion to human population density and pruning by the 99th percentiles of the serial interval and dispersal kernel. The effects of pruning assumptions and uncertainties on reconstructed transmission chains are shown in Figure D. 4 and an animation of the consensus transmission tree is provided as Movie D. 1

5.5 Discussion

From investigating this outbreak in a formerly rabies-free province, we identified at least three independent introductions that led to rapid island-wide spread. Although 46 animal cases and two confirmed human deaths were detected over the first 12 months, our inference suggests considerable transmission occurred prior to outbreak detection. Decreased dog vaccination coverage associated with the COVID-19 pandemic and long-distance human-mediated translocations likely increased the likelihood of both rabies introductions and spread.

Each year, around 200-300 people die of rabies in the Philippines (Department of Health & Department of Agriculture Bureau of Animal Industry, 2019). Mass dog vaccination is effective for rabies control and has been employed nationwide at varying consistencies. One successful local example is Bohol Province's intersectoral elimination program, which achieved 70% coverage (as recommended by WHO) through "catch-up" vaccination following mass campaigns (Lapiz et al., 2012). Models predict that vaccinating at least 60% of dogs should substantially reduce cases (Cleaveland et al., 2003), but if coverage is heterogeneous, time to elimination increases, while the probability of elimination decreases (Ferguson et al., 2015). Prior to 2020, vaccination coverage never exceeded 40% in Romblon due to budget and labour shortages, while poor coordination between municipalities led to patchy campaigns that lacked island-wide coverage. Herd immunity was likely low across much of the country, as vaccinations were suspended due to COVID-19 lockdowns in 2020 and 2021. Nationwide, human rabies deaths increased to more than 350 per year (Smith et al., 2024).

Our work supports previous findings that incursions occur frequently, with genomic surveillance revealing higher rates than expected (Volz et al., 2013). Many introductions fail to take off due to stochasticity in rabies transmission (Bourhy et al., 2016). However, recent outbreaks in other provinces such as Ilocos, as well as the formerly rabies-free island Marinduque, suggest large-scale re-emergence in the aftermath of COVID-19. The third cluster in Romblon was detected in a municipality with a ferry port, indicating an increase in inter-island introductions as travel heightened and restrictions relaxed. The Philippines' archipelagic nature may limit incursions in geographically isolated islands, and residual vaccination coverage reduces the chances of secondary cases. However, accessibility to nearby rabies-endemic provinces, coupled with poor vaccination coverage, likely contributed to the outbreak spread.

Prior to the pandemic, gaps in the province's surveillance were apparent: few samples were submitted annually, as storage in the Provincial Veterinary Office and transport by ferry were necessary for confirmation. With staff shortages, timely transfer to the regional laboratory was further impacted by the infrequency of ferry trips due to inclement weather. Apart from causing the suspension of vaccinations, which left dog populations vulnerable, COVID-19 restrictions hindered surveillance, delaying outbreak detection and possibly leaving earlier outbreaks undetected. Social distancing and prioritised pandemic response impeded investigation of two suspect deaths on Romblon Island in 2020. Samples from animals involved in high-risk bites were mostly not collected prior to the first RDT-positive case, but the result sparked multiple investigations, leading to increased sample collection and RDT use. News of the case result also catalysed testing of two samples that had been stored for over a month. These laboratory-confirmed cases proved that rabies had been circulating earlier than initially presumed.

Between 2017 and 2019, all submitted animal specimens from Romblon tested negative for rabies and no human deaths were reported, reaffirming the province's rabies-free status. Three and zero submissions in 2020 and 2021 respectively, were attributed to lockdown restrictions, while increased case detection and real-time investigation of the outbreak from 2022 onward were enabled by IBCM. Improved communication between animal and human sectors led to identification of most cases, through animal investigations that were triggered by bite victim reports. Use of RDTs (Cruz et al., 2023; Tenzin et al., 2020; Yale et al., 2019) may have compelled speedier investigations, since dissemination of positive RDT results increased awareness, which spurred immediate follow up of animals involved in biting incidents. Through these timely investigations, animal health workers collected suspicious animals that had died, and the upsurge in testing produced more confirmed cases. To compensate for Romblon's lack of laboratory facilities, multisectoral inter-island collaboration between provinces streamlined the sample transport process for confirmatory testing and subsequent sequencing. But the volume of samples highlighted challenges, exceeding available resources required to send them individually. Thus, all samples, whether untested, RDT-positive or RDT-negative, were forwarded in weekly batches for laboratory confirmation. When the regional laboratory could not perform DFAT, RDTs were performed instead, before transfer to a third location–the national laboratory (RITM)–for DFAT confirmation and sequencing, requiring additional travel time and exacerbating delays to result reporting.

Sequencing has played a crucial role in informing sources of rabies introductions (Mahardika et al., 2014; Sabeta et al., 2013; Trewby et al., 2017), and mobilising vaccination responses (Bourhy et al., 2016; Zinsstag et al., 2017). Integrating genomic data with epidemiological data from IBCM enhanced understanding of the outbreak spread and identified possible points of introduction, also suggesting the need for preventive vaccination, targeting dog vaccination towards source endemic areas. The benefits of genomic surveillance, as evidenced during the COVID-19 pandemic, require that expertise and skills are maintained in-country (Lee et al., 2022). Applying these methods for rabies can help build and sustain capacity for outbreaks of other emerging diseases, as what happened during the regional meeting with provincial veterinarians 3 months after the outbreak.

Veterinary capacity remains limited across much of Southeast Asia. In Tablas, few staff had to contend with the rapidly evolving public health emergency. The concurrent emergence of ASF prompted government-mandated enhanced surveillance across several provinces, including testing, culling, and banning importation of pork products from affected islands. In comparison, rabies outbreak response was decentralised, fragmented, differing between municipalities, and limited in scale. Prioritisation of ASF by the animal health sector set back investigations, and case confirmation delays slowed the public health response. No formal declaration of an island-wide outbreak was made, and while few municipalities declared a state of emergency, rabies control was limited. With insufficient dog vaccines and vaccinators, reactive coverage through ring vaccinations of <100 dogs following case confirmation may have provided a small radius of immunity but did little to contain the outbreak, as cases in neighbouring villages showed that transmission was already occurring across shared borders by the time of detection. Moreover, small-scale vaccination is known to be ineffective when only a proportion of cases are detected (Townsend et al., 2013a) (Cleaveland et al., 2003). Contact tracing and PEP prevented human deaths from confirmed animal cases, but poor awareness hindered contact tracing for probable cases, as some individuals refused PEP after consuming dog meat, fearing legal consequences. The local IEC activity conducted after a rabies-positive case had little impact on PEP-seeking behavior and the reporting of suspicious animals, as a human death occurred just two months later. Both human cases reportedly turned to tandok instead of PEP, and medical care was only sought after symptoms appeared.

These deaths could have been prevented if communities had been more aware of the rapid rabies spread already evident by December 2022. The fact that intensified response efforts only followed a human fatality – and that island-wide dog vaccination was not resumed until

2024, two years after the outbreak's detection – highlights the ongoing reliance on reactive rather than proactive health strategies, posing a significant One Health challenge.

Our study had several limitations, beginning with IBCM training and support being compromised by COVID-19 restrictions. Despite triggering the outbreak investigation, RDTs were challenging to incorporate into case finding, for several reasons. There was a two-year gap between RDT training and field deployment. Romblon's rabies-free status may have also created a false sense of security, explaining the lack of immediacy in testing suspicious animals. Lack of practice and confidence were reflected in samples that were stored post-collection, with the expectation that the regional laboratory would handle testing. Furthermore, positive RDTs were not considered valid unless matched by DFAT, so there was little incentive to use RDTs as they did not 'count' as a diagnostic method, even if waiting for laboratory confirmation delayed information dissemination. National authorization for the use of RDT and release of official diagnostic results could have expedited early outbreak detection, and if RDTs were recommended internationally, this could perhaps hasten implementation of control measures. Though it must be noted that laboratory confirmation still did not spur outbreak response in several municipalities until a human case was reported.

Genomic surveillance revealed insights not possible from the epidemiological data alone but were not definitive. For example, the human case sequence points to a second introduction from an unknown source that we were unable to pinpoint, due to undersampled diversity in the phylogeny. As this sample type (skin) and extraction kit was not ideal (sequencing approaches have been optimised for brain tissue), this sample might need revisiting to generate better sequence coverage and depth. Moreover, not all positive samples were sequenced, with only 24/43 early samples sequenced to date. Sequences from later in the outbreak could reveal which lineages have persisted and if further introductions have occurred. The largest cluster likely resulted from a single introduction from Bulacan province that diverged into three genetic lineages, but it could have resulted from multiple introductions. However, without more sequences from this period and from Bulacan province, we are unable to distinguish these scenarios. Our inference of orphaned cases and short transmission chains indicate either locations with undetected transmission, or long-distance (human-mediated) translocations. Longer delays make our branching process approximation less accurate for estimating undetected transmission, as observed for the largest cluster associated with the initial outbreak detection. Further methodological

development could refine these estimates, including accounting for uncertainty in the timing of introductions and for residual vaccination coverage.

Free-roaming dog populations sustain rabies outbreaks worldwide, as seen in Romblon, where most cases were from owned dogs that were unleashed and unsupervised. It is a cultural practice in some LMICs to let dogs wander, and despite local ordinances in the Philippines prohibiting non-leashed dogs in public, these are not easily implemented due to insufficient resources for dog-catching and impounding. Therefore, the burden must also be shared with dog owners to take responsibility for ensuring that their pets are vaccinated and not inconveniencing others.

Achieving vaccination coverage of 70% remains the most important rabies control method. Dog vaccination estimates extrapolated from different sources showed potential inconsistencies (Figure **5.3**A), and heterogeneous coverage in the Philippines is evidenced by lack of coordination among municipalities, even during a deadly outbreak on a small island. If one municipality achieves sufficient coverage, it is still vulnerable to incursions from neighbouring municipalities, highlighting the value of cross-border coordination. Similarly, focus must be placed on proximate control measures in nearby rabies-endemic islands if the 'Zero by 30' goal is to be achieved.

5.5.1 Conclusions and recommendations

This investigation demonstrates the value of combining epidemiological and genomic data for inferring the source and spread of rabies outbreaks. Enhanced surveillance through IBCM coupled with genomic surveillance proved essential in case-finding and tracking, while simultaneously highlighting the challenges of outbreak detection and response in rural archipelagic settings. The immediacy of RDT results illustrate their potential to inform timely outbreak declaration and response, but lack of international guidance on their use remains an obstacle.

Despite belying the One Health approach, control measures driven solely by human deaths are unfortunately common in LMICs, with dog rabies cases often not taken seriously. Lessons should be taken from Romblon on RDTs and laboratory-confirmed animal cases acting as triggers for outbreak response. The Philippines has previously demonstrated rabies control capacity, but since its economic impact is negligible compared to ASF (despite human fatalities), routine surveillance remains neglected and border control measures have

not been strengthened amidst disease re-emergence. Delayed public health responses that included small-scale ring vaccinations were inadequate, emphasising the need for dog vaccination to be sufficiently large-scale, in this situation, island wide. Genomic surveillance is beneficial for determining the source of incursions and can also target preventative vaccination toward rabies-endemic areas. Additionally, sustaining genomic capacity can benefit investigations of other infectious diseases in human and animal populations, with rabies serving as a marker of government response proficiency. Globally, lessons from this outbreak include proven benefits of the One Health approach in enhancing surveillance, the limitations of short-term control measures, and the importance of routine surveillance in maintaining capacity for responding to potential re-emergence.

Chapter 6

General Discussion

Infectious disease elimination refers to the reduction of disease transmission to zero in a specific geographical area or population through targeted interventions, public health strategies, and sustained efforts (Dowdle, 1998; Klepac et al., 2013). Achieving elimination requires not only effective intervention but also continuous monitoring to detect and respond to any resurgence of cases. The global goal 'Zero by 30' sets a clear target to eliminate dog-mediated human rabies deaths by 2030 through coordinated mass dog vaccination, enhanced surveillance, public education, and timely post-exposure prophylaxis.

Surveillance is an indispensable tool in the elimination of infectious diseases like rabies, providing information for early detection, outbreak investigation, strategic public health responses, and for monitoring outcomes of control measures to inform changes (Townsend et al., 2013a). Epidemiological surveillance, often called "information for action" involves systematically collecting, analysing, and interpreting data about who becomes infected, where, when, and under what circumstances (CDC, 2023). This information helps guide immediate responses and long-term policy decisions.

Genomic surveillance on the other hand uses genetic sequencing of pathogens to track the evolution, spread, and emergence of new variants (Gardy & Loman, 2018). The COVID pandemic has demonstrated the high impact of genomic surveillance in understanding transmission dynamics and for an effective outbreak response strategy. Prior to this study, genomic surveillance in the Philippines was limited, with most molecular work focused on partial gene sequences and minimal integration into national surveillance programs. This thesis demonstrated the critical role of genomic sequencing in advancing global rabies elimination efforts and its practical application in LMIC settings. It also highlighted how integrating genomic and epidemiological data strengthens rabies surveillance by providing deeper insights into patterns of disease spread, lineage persistence, and outbreak origins.

In this chapter, I discuss the key findings of my thesis and the practical implications for improving rabies surveillance systems, as well as the challenges and limitations of integrating genomics into routine public health practice aimed at achieving rabies elimination.

6.1 Genomic Surveillance Potential

This thesis highlights the potentially valuable role of genomic sequencing in advancing global rabies elimination efforts. Genomic data when integrated with epidemiological data,

can trace sources of infection, investigate host shifts, detect transboundary spread and incursions, and guide targeted control strategies. These are functions have been successfully demonstrated in studies of other zoonotic pathogens, such as Ebola (Carroll, 2019; Dudas et al., 2017; Mbala-Kingebeni et al., 2019; Quick et al., 2016) and SARS-CoV-2 (Andersen et al., 2020; Chen et al., 2022; Rockett et al., 2020; Sahadeo et al., 2023), where genomic surveillance significantly enhanced outbreak response and containment. However, despite its potential for advancing disease control, genomic sequencing remains underutilized in many rabies-endemic regions, where its application is often limited to academic research rather than incorporated into public health programs, as highlighted in Chapter 2. This limited adoption may be attributed to several implementation barriers, including reliance on external funding through project collaborations abroad (Getchell et al., 2024), supply chain challenges that affect reagent and equipment availability (Brunker et al., 2020), shortages of trained personnel, both in laboratory and bioinformatics aspects of genomic sequencing (Onywera et al., 2024). Chapter 3 aims to support adoption of lower cost and practical approaches to genomic surveillance for countries like the Philippines, but these challenges remain difficult to overcome. Furthermore, genomic surveillance is often not prioritized in favour of potentially more immediate or cost-effective public health interventions, as it is perceived to be technically complex and resource intensive.

A limitation of this study is the lack of a detailed cost analysis comparing the implementation of genomic sequencing with other public health priorities such as dog vaccination, health education, or bite management programs. The cost-benefit of allocating limited resources to sequencing, particularly when these resources could otherwise support high-impact interventions were not evaluated. Nonetheless, a major contribution of this study is the development of a rapid, cost-effective nanopore sequencing workflow tailored for low-resource settings. This workflow provides a practical tool for characterizing rabies viruses and helps address common barriers such as high costs, limited infrastructure, and the need for specialized technical expertise. In many low-resource settings, the decision to invest in genomic technologies must be weighed against essential frontline interventions, and the opportunity cost of diverting funds from more immediately impactful measures such as mass dog vaccination campaigns require careful consideration. While genomic sequencing may not offer immediate benefits comparable to vaccination, its value lies in long-term gains—such as improved outbreak detection, better understanding of transmission patterns, and more effective, targeted responses. A comparative cost-benefit analysis would help guide such decisions and support the integration of sequencing into routine programs, especially in contexts where health budgets are constrained. However, it should be noted that

sequencing costs are additional to general improvements for surveillance which are expected to increase detection of cases but also incur costs for field activities and diagnostics (such as the application of IBCM described in Chapter 5). Cost-benefit questions of additional sequencing are therefore nuanced in relation to how effective standard epidemiological surveillance is in guiding interventions, and what additional information is gained from genomic insights, which will be highly contextually dependent.

Experience of developing approaches for genomic sequencing of rabies in the Philippines, and in providing training to practitioners, both from the Philippines and from other parts of the world provides lessons for future deployment. Given the current challenges in the Philippines, establishing sustainable rabies genomic surveillance will require strategic planning, and strong political commitment. Key recommendations to enable more effective response to rabies and other infectious diseases should be considered: 1) Building capacity in genomics and bioinformatics among local researchers and public health professionals; 2) Upgrading diagnostic laboratory facilities to support high-throughput genomic sequencing with good laboratory practice; 3) Strengthening bioinformatics infrastructure and 4) Promoting collaboration between government agencies, academic institutions, and international partners; 5) Securing consistent government funding; 6) Integrating genomic surveillance data into public health decision-making with clear protocols for translating genomic findings into actionable strategies for disease control and elimination; 7) Increasing awareness among policymakers and the public of the importance of genomic surveillance in disease prevention and control.

Specifically in the context of genomic surveillance of RABV, my review in Chapter 2 identified the need for standardized classification methods and phylogeny-based nomenclature. This would help improve understanding of patterns of RABV circulation, including the source of introductions, learning for approaches developed during the COVID-19 pandemic. The comprehensive, publicly available protocol I present in Chapter 3 covers sample collection, sequencing, and data analysis, suited for low-resource settings and includes user-friendly bioinformatics tools for rapid data interpretation, even for users with minimal bioinformatics experience, and a phylogenetic classification tool to standardize rabies virus nomenclature. These approaches provide a strong basis for helping to routinize genomic surveillance for RABV and overcome some of the barriers described earlier.

6.2 Inferring Rabies Dynamics from Virus Sequencing

Focusing on finer-scale temporal patterns and evolutionary history, the analysis of all available genetic data used in Chapter 4 (publicly available data, as well as the extensive WGS dataset produced by my study) revealed strong geographic associations by island group (Luzon, Visayas, and Mindanao) with increased inter-regional and inter-provincial lineage mixing. The unique geographic landscape of each island group influenced RABV movement over time. In Luzon, vast plains and populated valleys likely contributed to the spread of rabies, reflected in its higher genetic diversity compared to other island groups. In contrast, the seas and mountain ranges in the Visayas and Mindanao, respectively, have restricted the movement of infected animals, limiting the spread of RABV. This aligns with previous studies showing that geographic features and natural barriers such as mountains, rivers, and seas play crucial roles in the containment and spread of rabies (Brunker et al., 2018; Coetzer et al., 2017b; Muleya et al., 2019; Talbi et al., 2010; Yc et al., 2016; Zhao et al., 2019).

Although the spread of rabies across island communities is strongly shaped by geographic isolation, in Chapter 5 I identified three independent introductions of rabies into a previously rabies-free island in the Philippines, all originating from nearby endemic provinces. These incursions are attributed to human-mediated movement of infected animals, compounded by reduced vaccination coverage and relaxed border controls. The situation was further exacerbated by the global disruption of essential health services particularly for neglected diseases like rabies during the COVID-19 pandemic as observed in other endemic areas (Nadal et al., 2022). Similar outbreaks in other provinces point to a large-scale post-pandemic resurgence of rabies, largely driven by the interruption of routine control measures, specifically mass dog vaccination. These findings are consistent with rabies outbreaks reported in other island communities such as in Bali, Indonesia (Mahardika et al., 2014), Malaysia (Faizul et al., 2019), Timor-Leste (Amaral Mali et al., 2024) and on Pemba Island, off the coast of East Africa (Lushasi et al., 2023), where incursions were traced back to introductions from nearby populations.

The use of WGS in this study provided higher phylogenetic resolution and stronger posterior support compared to partial genome sequencing, which showed limited resolution and greater uncertainty in estimating divergence times. This advantage was particularly evident in the outbreak investigation presented in this thesis (chapter 5), where WGS—combined with enhanced surveillance through IBCM enabled more precise determination of the timing

and source of virus introduction. However, despite these insights, the outbreak continued to spread within the island community for over a year. This persistence was largely due to challenges in translating research findings into actionable policies including the lack of available vaccines needed for outbreak response. Contributing factors that prevented containment included weak enforcement of existing control measures, a shortage of dedicated animal and public health workers for rabies control, limited funding as rabies was deprioritized in favor of high-impact livestock diseases like African Swine Fever (ASF) and Avian Influenza, and the fragmented management of zoonotic diseases across sectors. In the Philippines, China and Africa (chapter 2) compared to other endemic countries like Vietnam,Thailand, and Nigeria, a great deal of whole genome sequencing of rabies viruses has been undertaken, but still lacks the large database that other pathogens have like Influenza (Global Influenza Surveillance and Response System) (*Global Influenza Surveillance and Response System (GISRS)*, 2024), Malaria (MalariaGEN) (*MalariaGEN*, 2024), Antimicrobial resistance (Global Antimicrobial Resistance Surveillance System) (*Global Antimicrobial Resistance and Use Surveillance System (GLASS)*, 2024), and COVID-19 (Nextstrain) (Hadfield et al., 2018).

One limitation of this study is the uneven availability and representativeness of genetic data across geographic and temporal scales. Although the study combined publicly available sequences with a newly generated whole-genome dataset, some provinces or time periods were still underrepresented, potentially biasing interpretations of lineage movement and viral diversity. This can affect the ability to detect all transmission routes or fully resolve the evolutionary history of circulating RABV lineages. Additionally, while whole-genome sequencing provided improved phylogenetic resolution, the lack of detailed metadata (e.g., precise sampling dates, host movement history, and vaccination coverage) limited the integration of genomic findings with epidemiological dynamics. This highlights the challenge of recording metadata accurately and the importance of addressing this challenge in a LMIC like the Philippines. Furthermore, conclusions about the influence of geographic barriers on virus spread are inferred from genetic patterns and may not fully capture the complexities of human behavior, animal movement, and surveillance coverage, which also significantly shape rabies transmission. These limitations highlight the need for more comprehensive, standardized genomic surveillance and better integration with contextual field data**.**

Genomic sequencing can play a pivotal role in identifying RABV diversity to formulate strategic island-specific control measures for rabies. Tailored to each island group where

some degree of compartmentalization and transboundary transmission was observed, control efforts should aid in the ultimate goal of zero human death by 2030. Policies on strict border control in smaller island groups and coordinated vaccination strategies with consideration of shared land borders in bigger island groups could be initiated for more effective rabies control. Further phylogeographic analysis will enhance knowledge of viral spread and population dynamics, informing future rabies control efforts. The limitation of sampling bias could be mitigated by integrating partial and whole genome sequences, as demonstrated by Holtz et al (Holtz et al., 2023), for a more comprehensive analysis of virus evolution and transmission.

6.3 Future directions for genomic surveillance and application towards rabies elimination

A major challenge for researchers lies in presenting their findings and recommendations in an accessible manner without compromising scientific integrity. Effective communication of research outcomes is essential for informing policymakers and shaping control and elimination strategies. This challenge is also true with rabies control and elimination despite the Philippines taking significant steps to combat rabies as a neglected tropical disease. The complex interplay of ecological, biological, and socio-economic factors. To sustain progress toward the goal of zero human deaths from rabies by 2030, international cooperation and coordinated rabies control efforts are needed to reduce the transboundary spread of rabies virus and limit reintroductions (Lucien et al., 2023; Sahadeo et al., 2023). This is a consistent and clear message that came out in several of my chapters (2, 4 and 5).

Although the Philippines has adopted the global "Zero by 30" framework, the persistence and recurrence of outbreaks, particularly in previously declared rabies-free provinces like Marinduque, Romblon, and Bohol, highlight difficulties in maintaining long-term rabies-free status, especially after disruptions caused by COVID-19 pandemic. This thesis emphasizes the vital role of genomic surveillance in addressing these persistent challenges.

A One Health approach, utilizing enhanced surveillance methods such as Integrated Bite Case Management (IBCM), has proven effective in zoonotic disease management in other endemic countries, like Tanzania (Brunker et al., 2012) and India (Gibson et al., 2022). This thesis demonstrated how IBCM facilitated timely detection and response during a rabies outbreak but was not fully optimized due to challenges linked to cultural, intrinsic, and socio-economic factors, as well as the challenges of implementation during the COVID-19

pandemic. In the Philippines, rabies outbreaks in the MIMAROPA region showcased how IBCM uptake can vary, with positive effects on intersectoral coordination during outbreaks. The Romblon outbreak, discussed in Chapter 5, marked a turning point in improving the region's outbreak investigation and response efforts. Lessons learned from Romblon significantly enhanced how a subsequent outbreak in Marinduque, also in the MIMAROPA region, was managed.

In Chapter 5 I demonstrated how genomics can be integrated into rabies surveillance by utilizing phylogenetic analysis of outbreak sequences to pinpoint the origin and timing of virus introductions. This crucial information prompted stakeholders to take coordinated action, leading to successive vaccination campaigns across the MIMAROPA region in response to the outbreaks. These efforts align with the findings from Chapter 4, which emphasize the importance of region-specific targeted controls, especially in addressing incursion events.

This thesis establishes the foundation for integrating enhanced genomic surveillance into National Rabies Prevention and Control Program (NRPCP) strategic framework. A fully operational genomic sequencing workflow tailored for LMICs is provided in Chapter 3. The phylogenetic analysis in Chapter 4 demonstrates the pivotal role of genomic sequencing in developing targeted island-, region-, and province-specific control measures for a more efficient and effective strategy. Chapter 5 served as a case study showing how integrating genomic and epidemiological data enhances outbreak responses. The adoption of IBCM confirmed that a One Health approach can overcome challenges in eliminating rabies and other zoonotic diseases, supporting the global "Zero by 30" goal. In this way, my thesis illustrates the value of genomic surveillance for rabies control and recommends for its full integration, aligning with key strategies for improving governance, service delivery, regulation, and capacity building in rabies elimination efforts.

Promising future research directions that have emerged from this thesis include the following: 1) Establishing more comprehensive genomic databases for rabies, including especially more viruses from underrepresented areas, to better track viral migration and outbreaks in real-time, 2) Investigating how geographic and ecological barriers influence the genetic diversity and spread of rabies, 3) Exploring the impact of cultural practices, pet ownership, and public awareness on the success of vaccination campaigns and other control measures, 4) Applying genomic methodologies used in rabies research to other pathogens, 5) Developing faster and more accurate diagnostic tools for broader zoonotic disease

prevention. These directions hold the potential to advance strategies for control of rabies and other zoonotic diseases, with the possibility of extending efforts within Southeast Asia, towards a coordinated regional approach to genomic surveillance.

Appendix A:

Chapter 2 Appendix

A.1 Supplementary Files

Table A. 1. Clade designations from various publications and their corresponding RABV-GLUE classifications.

Country	Number of papers reviewed	Paper_designation	RABV-GLUE designation
Afghanistan	*	Arctic-like lineage	Arctic AL1b
Azerbaijan	2	Cosmopolitan lineage	Cosmopolitan Cosmopolitan ME1a Cosmopolitan CA2
Bangladesh	1	AAL2	Arctic AL1a
Bhutan	1	Arctic-like-1	Arctic Arctic AL1a
Cambodia	1	SEA1 SEA2 SEA3	Asian SEA3
China	55	Asian 1=1a,	Asian SEA1a
		China I	Asian SEA1b
		Clade 1: IA and IC subgroup	Asian SEA1b
		Clade I of lyssavirus genotype I	Asian SEA1b
		Clade I: Lineages A, B, C, D, E, F	Asian SEA1b
		Genotype 1, subgroup A	Asian SEA1b
		SEA1	Asian SEA1b
		Asian 2	Asian SEA2a

		China Clade II	Asian SEA2a
		Genotype 1, subgroup B	Asian SEA2a
		SEA2	Asian SEA2a
		China II	Asian SEA2b
		Clade 2	Asian SEA2b
		Clade B	Asian SEA3
		China I-VII: China IV	Arctic AL2
		China clade III	Cosmopolitan
		China I-VII: Clade III	Cosmopolitan
		China I-VII: China VII	Indian Subcontinent
		China I-VII: Clade I, II, V, VI	Asian SEA1b, Asian SEA2: a&b, Asian SEA1a, Asian SEA3
		Group I, II, III <J84>	Asian SEA1b, Asian SEA2a, Cosmopolitan
		Group I, II, III, IV <J83>	Asian SEA2a, Asian SEA1b, Asian SEA3, Cosmopolitan Vac
		Lineage A, B, C1, C2 <J79>	Asian SEA1b, Asian SEA2a, Cosmopolitan, Asian SEA1b
		6 Lineages: A, B, C, D, E, F <J98>	Cosmopolitan, Arctic AL2, Asian SEA1b, Asian SEA1a, Asian SEA2: a&b, Asian SEA3
		Group I: YN-A (China I), YN-B (ChinaVI), YN-C (ChinaII), YN-D (China III)	Asian SEA1b, Asian SEA3,

			Asian SEA2a, Cosmopolitan
		6 FB-related lineages: FB I, II, III, IV (China II); FB V, VI (China I)	Asian SEA2b; Asian SEA1b
Georgia	*	Cosmopolitan	Cosmopolitan CA2
India	13	Indian Lineage I; Indian Lineage II	Arctic, Arctic AL1a; Asian SEA1a, Indian sub
		GC1	Arctic AL1a
		Cluster I	Arctic AL1a
		Gr-1: Gr-1A, Gr-1B	Arctic AL1a, Arctic AL1a
		4Clusters: Cluster 1, 2, 3, 4	Arctic AL1a, Arctic AL1a, Indian-sub, Cosmopolitan
		Gr-I/Arctic like lineage; Gr-II /Asian lineage	Arctic AL1a; Cosmopolitan Vac
		IN-1= Northern India, IN-2=Southern India	Arctic AL2, Arctic AL1a
		5 Genetic clusters: GC 1,2; GC 3,4,5	Arctic, Arctic AL1a; Arctic AL1b
Indonesia	4	descendant of Kalimantan strain ID1; ID2; ID3 2 major clusters: Group I and Group II 3 lineages: Lineage 1(Sumatra), 2(Flores), 3(all rabvs from Bali)	Asean SEA1b
Iran	3	Group 1; 2a,2c; 3	Cosmopolitan CA2; Cosmopolitan ME1a, Cosmopolitan CA1; Arctic
Iraq	3	Cosmopolitan	Cosmopolitan CA2, Cosmopolitan ME1a,

Israel	2	Variants I-VII: Israel I-V; VI; VII	Cosmopolitan ME1a; Cosmopolitan ME1b; Cosmopolitan ME2
Jordan	1	Cosmopolitan ME1 Cosmopolitan ME2	Cosmopolitan ME1a Cosmopolitan ME1b
Kazakhstan	1	Cosmopolitan	Cosmopolitan Cosmopolitan CA1
Korea	4	Arctic Gangwon variant Arctic lineage: A & B	Arctic AL2
Laos	1	SEA: Laos II Lineage Laos III Laos IV	Asian SEA3
Malaysia	1	Asian I Asian II lineage	Asian SEA1b Asian SEA3
Mongolia	4	Group A; Group B	Cosmopolitan CA1; Arctic AL2
		Clade1(Asian), Clade 2(Cosmopolitan)	Asian SEA1b; Cosmopolitan CA1
		Subclade A; Subclade B	Cosmopolitan CA1
Nepal	1	Indian Subcontinent Arctic Like: AL1 and AL3	Arctic AL1a Arctic AL3 Cosmopolitan Vac Indian-Sub
Oman	2	Genotype 1	Cosmopolitan ME1a
Pakistan	*	Arctic-like lineage	Arctic AL1a Arctic AL1b

Philippines	8	Classical rabies virus (genotype I) Grp 1, Grp 2 Clades and subclades: Group L=Luzon (GrL1-9; GrSL, GrMD) Group V= Visayas (V1, V2) Group M=Mindanao Asian 2b cluster with 3 clades: Luzon, Visayas, Mindanao SEA4 Clade 1,2,3	Asian: SEA4
Qatar	1	Cosmopolitan	Cosmopolitan ME1a
Russia	4	Eurasian group, N-E Northeastern group; C-R Central Russian group; Cau- Caucasian group	Cosmopolitan CA1; Cosmopolitan NEE; Cosmopolitan CA; Cosmopolitan CA2
Saudi Arabia	*	Cosmopolitan	Cosmopolitan ME1a
Sri Lanka	2	Independent lineage: Indian Sub Clades I-VII	Indian Sub
Taiwan	5	TWI and TWII: SEA5 3 major genotypes: Eastern; Central; Southern RABV-TWFB Sub cluster I, II, III	Asian SEA5
Thailand	5	Clades 1-6	Asian SEA3
		T1 and T2 with 4 lineages each	Asian SEA3
		THA-1A; THA-1B; THA-2	Asian SEA3
		TH1: A, B, C; TH2: A-H	Asian SEA3
Turkey	2	Clade 1; Clade 2	Cosmopolitan ME2

United Arab Emirates	*	Cosmopolitan	Cosmopolitan ME1a
Vietnam	2	SEA 1	Asian SEA3
		Grp 1: subgrp 1a and 1b; Grp 2	Asian SEA3; Cosmopolitan, Cosmopolitan Vac
Algeria	1	AF1a	Cosmopolitan AF1a
Botswana	1	AF1b	Cosmopolitan AF1b and AF3
Brazil	19	AgV1 & AgV2	Bats DR, Cosmopolitan AM3a, Cosmopolitan AM3b, Cosmopolitan,
		Subgroup I and IIa, IIb, IIc assigned geographically	Cosmpolitan AM3a and Cosmopolitan AM3b, Cosmopolitan AM3b, Cosmopolitan
		Cluster I and Cluster II, assigned geographically	Bats DR and Bats DR
		Domestic clade and Wild clade	Cosmpolitan AM3a and Cosmopolitan, Cosmpolitan AM3a
		PB Foxes, PB Insectivorous bats, PB Cattle	Cosmpolitan AM3b
		AgV1 (vampire bat related)	Bats DR
		Clade I; grp A, B, C and Clade II: grpD, E	Cosmpolitan AM3b, Cosmpolitan AM3a, Bats DR and Bats DR, Bats DR
		Domestic (cluster 1A, 1B) and Wild (cluster 2A, 2B)	Cosmpolitan AM3a, Cosmpolitan AM3a and Cosmpolitan AM3b, Cosmpolitan

		Bats Cluster and Canids Cluster	Bats DR, Bats TB2, Bats and Cosmopolitan, Cosmopolitan AM3a
		Lineage I and Lineage II	Bats DR and Cosmopolitan AM3a
		Cluster I, II, III	Bats DR, Bats, Cosmopolitan AM3a
		Two clusters - Chiroptera and Carnivora: Lineage A, B	Bats DR and Cosmopolitan AM3b, Cosmopolitan AM3a
		Cluster I, II and III: Subcluster 3.1, 3.2, 3.3	Cosmopolitan AM3a, Cosmopolitan AM3b and Cosmopolitan, Cosmopolitan AM3b, Cosmopolitan AM3b
		Group A& B, assigned both geographically and by host (dog and bat)	Bats DR, Cosmopolitan AM3a, Cosmopolitan AM3b, Cosmopolitan,
		5 lineages (A-E) according to the host and the source of rabies	Bats DR, Cosmopolitan AM3a, Cosmopolitan AM3b, Cosmopolitan,
		Dog and vampire bat related	Bats DR, Cosmopolitan AM3a, Cosmopolitan AM3b, Cosmopolitan,
		Two group - carnivora and chiroptera	Bats DR, Cosmopolitan AM3a, Cosmopolitan AM3b, Cosmopolitan,
Burkina Faso	1	AF2	AF2
Cameroon	1	AF1 (split into 2 lineages but not as per RABV-GLUE), AF2	Cosmopolitan AF1a, Cosmopolitan AF1b, AF2

Central African Republic	2	AF1b and 2	Cosmopolitan AF1b and 2
Chad	2	AF1 and 2	Cosmopolitan AF1a, Cosmopolitan AF1b and AF2
		AF1	Cosmopolitan AF1a, Cosmopolitan AF1b and AF2
Colombia	3	AgV1 & AgV2	Cosmopolitan AM2a, Bats DR,
		Three groups - domestic dogs, insectivorous bat and hematophagous bat	Cosmopolitan AM2a, Bats DR,
		AgV1 & AgV4	Cosmopolitan AM2a, Bats DR,
Ethiopia	4	AF1a	Cosmopolitan AF1a
		AF1a	Cosmopolitan AF1a
		AF1a	Cosmopolitan AF1a
		AF1a	Cosmopolitan AF1a
Georgia	1	Cosmopollitan	Cosmopolitan CA2
Ghana	2	AF1(a and b) and AF2	Cosmopolitan AF1a, Cosmopolitan AF1b and AF2
		AF1(a and b) and AF2	Cosmopolitan AF1a, Cosmopolitan AF1b and AF2
Kenya	1	AF1(a and b)	Cosmopolitan AF1a and Cosmopolitan AF1b
Lesotho	1	AF1b (divided into clade A, B and C)	Cosmopolitan AF1b
Liberia	1	AF2	AF2
Malawi	1	AF1b	Cosmopolitan AF1b

Mali	1	AF2 (divided into clades F, G and H)	AF2
Mexico	5	Insectivorous bats	Bats TB1, Cosmopolitan Vac2
		Two groups: domestic dogs and skunk rabies	Bats TB1, Cosmopolitan Vac2, Cosmopolitan AM2a
		AgV1	RAC-SK
		AgV1	Bats TB1, Cosmopolitan Vac2
		AgV1 & AgV7 and skunk	Cosmopolitan AM2a, AM2b, Cosmopolitan Vac2, Cosmopolitan and RAC-SK
Mozambique	1	AF1b (divided into clade A, B and C)	Cosmopolitan AF1b
Namibia	2	AF1b	Cosmopolitan AF1b
		AF1b	Cosmopolitan AF1b
Nigeria	3	AF2	AF2
		AF2	AF2
		AF2	AF2
Senegal	1	AF2	AF2
South Africa	16	AF1b	Cosmopolitan AF1b and AF3
		AF1b and AF3	Cosmopolitan AF1b and AF3
Sudan	2	AF1a	Cosmopolitan AF1a
Tanzania	10	AF1b	Cosmopolitan AF1b
		AF1(a and b)	CosmopolitanAF1A and CosmopolitanAF1b
Tunisia	2	AF1a	Cosmopolitan AF1a

Uganda	2	AF1a and b	Cosmopolitan AF1a and Cosmopolitan AF1b
United Arab Emirates	1	Cosmopolitan	Cosmopolitan ME1a
West Indies	1	AgV1	
Zambia	2	AF1b	Cosmopolitan AF1b
Zimbabwe	1	AF1b	Cosmopolitan AF1b

* For countries which don't have a country paper but are included as part of a wider analysis e.g. Azerbaijan in the middle east paper

Table A. 2. Search terms used to select publications

Three search engines were used to select the articles for this review with search terms as shown.

Database	**Search terms**
PubMed	Rabies AND (genomic OR genome OR sequencing OR sequence OR molecular OR phylogenetic OR phylogeny OR phylogeography OR phylodynamic) AND (control OR surveillance OR elimination OR eliminate) NOT (Bat rabies)
Web of Science	(Rabies AND (genom* OR sequenc* OR molecular OR phylo*) AND (control OR surveillance OR eliminat*)) NOT (Bat rabies)
Google scholar	Rabies AND (genomic OR genome OR sequencing OR sequence OR molecular OR phylogenetic OR phylogeny OR phylogeography OR phylodynamic) AND (control OR surveillance OR elimination OR eliminate) NOT (Bat rabies)

A.1.1 Viral Sequencing to Inform the Global Elimination of Dog-Mediated Rabies - Protocol

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Running title / question – How can genetic surveillance support the elimination of rabies?

Abstract

Background

Rabies is a deadly yet neglected infectious disease. Present in almost 150 countries around the world, with most deaths reported in Asia and Africa, rabies is a serious pressing issue worldwide. A global strategy has been initiated with the aim of eliminating human deaths from rabies spread by domestic dogs by 2030 ('Zero by 30'). Genomic surveillance is a tool that can potentially support the 'Zero by 30' strategy.

Methods

The databases PubMed, Google Scholar and Web of Science will be searched to identify original studies published since the year 2000 with the following search terms 'Rabies AND (genom* OR sequenc* OR phylo* OR molecular) AND (control OR surveillance OR eliminat*)'. Pre-defined inclusion and exclusion criteria will be used to select relevant studies, and the selection procedure will be shown by a Preferred Reporting Items for Systematic reviews and Meta-analysis study flow diagram (PRISMA). Data will be extracted including author, year of publication, location of study, study design, sequencing platform, and coverage of genome (whole or partial), type and number of samples sequenced, infected host species, analysis methods, conclusion(s) of the study and any recommendations for control measures or surveillance derived from the genomic data. Data will be summarized in terms of trends in published papers, geographical coverage, sequencing platforms, length, and available genomic data.

Expected output

To get geographical coverage of the country with sequencing data in Africa to see where gaps are to expand the need of genomic surveillance. To give recommendations on how to improve genomic surveillance based on sample types, sequencing platforms, and data management. Key messages from study's conclusion regarding how genomic surveillance provides insights to inform Zero by 30 by drawing the unique message from genomic data.

Registration: This protocol will be submitted to the PROSPERO database for registration.

Keywords: Sequencing, phylogenetic, lyssavirus, molecular techniques

Background

Rabies Virus (RABV) poses the greatest public health threat, causing an estimated 60,000 deaths annually, almost all of which occur in Low- and Middle-Income Countries (LMICs) [1]. RABV is most commonly transmitted through bites from infected hosts in the orders *Chiroptera* and *Carnivora* [2]. Domestic dogs are the main source of transmission to

humans, but as a multi-host pathogen, wild carnivores also serve as primary RABV hosts with host-associated variants recorded in certain geographies [3]. For example, wildlife such as raccoons, skunk, and foxes each maintain different RABV variants in localities across North America [3]. Generally, RABV is referred to according to these host-associated variants (sometimes termed biotype, see definitions introduced in Box 1). Phylogenetic analysis enables further classification of RABV diversity into clades, subclades, and lineages, usually associated with specific geographic areas and/or hosts. The RABV genome is 12 kilobases (kb) in length [4], comprising five genes encoding the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large polymerase protein (L) [5]. Like other RNA viruses, RABV exhibits elevated mutation rates because of the absence of proofreading activity in the L protein [6]. Viral sequence data is informative because of these elevated mutation rates, which generate genetic diversity enabling improved tracking of viral spread and understanding of viral dynamics across space and over time.

There is no treatment for rabies once clinical signs begin, but post-exposure prophylaxis (PEP) administered shortly after a rabies exposure is almost 100% effective in preventing the fatal onset of disease [7]. Canine rabies elimination is possible through mass dog vaccination, as demonstrated in Europe, North America, parts of Asia and much of Latin America [8]. Several countries where dog-mediated rabies was previously endemic have now been declared rabies-free (Western Europe, Canada, the USA, and Japan) or are approaching elimination as a result of sustained dog vaccination [8]. According to the World Health Organisation (WHO), to eliminate dog-mediated rabies, vaccination campaigns need to achieve coverage of at least 70% of the dog population and be conducted annually for at least three years [9]. The incidence of rabies in Latin America has declined dramatically over recent decades due to coordinated regional elimination programs underpinned by this approach [8]. In contrast, most LMICs in Asia and Africa have not allocated sufficient budget to control this neglected disease. In these endemic countries, rabies surveillance is typically poor and challenges to rabies control include lack of understanding of dog ownership patterns, dog population sizes and dog accessibility for vaccination as well as cultural practices including dog meat consumption [10]. To address these challenges, international organisations recently joined forces under the United Against Rabies collaboration to advocate for the global goal of ‘Zero by 30’, to end human deaths from dog-mediated rabies by 2030 [11].

Surveillance plays a critical role in the control and elimination of infectious diseases [12]. Surveillance entails the continuous, systematic collection, analysis, interpretation, and timely dissemination of health-related information [13], serving as the foundation for planning, execution and evaluation of public health strategies. For instance, surveillance aids in producing data on the effectiveness of interventions, thus offering valuable insights for decision-making crucial for elimination initiatives like 'Zero by 30' [14]. Increasingly, surveillance involves genetic data, for pathogen diagnosis, for determining risks associated with a pathogen or its susceptibility to drugs, as well as to identify the source of outbreaks and to characterise pathogen spread [12]. Linked with locations, pathogen genetic data have uncovered different aspects of disease movement, from global migration dynamics to local transmission pathways for pathogens such as Influenza [15,16], Ebola [17], Zika [18], Yellow fever [19,20], Mpox [21–24] and SARS-CoV-2 [25]. Sequencing approaches have potential to enhance rabies surveillance and provide actionable information to inform rabies control programs locally and as part of 'Zero by 30'. For example, viral sequence data can distinguish continuous undetected local circulation from incursions and potentially identify their sources [26]. More generally, sequencing could provide key insights into how rabies circulates within different populations and the processes responsible for RABV maintenance in specific localities [12,27].

Use of pathogen sequence data within surveillance programmes is, however, not yet routine in most LMICs. Constraints include lack of local sequencing capacity, trained personnel and laboratory resources, affected by the costs of and access to reagents and consumables, as well as power supplies and cold chain [17]. Sequencing technologies have become more affordable, and efforts are underway to improve their accessibility [28]. Indeed, growth in sequencing capacity in LMICs during the COVID-19 pandemic provided evidence of the feasibility of scaling up molecular diagnostics but also highlighted operational challenges. For example, public health laboratories in Nigeria capable of molecular identification of SARS-COV-19 from clinical specimens increased from four to 72 laboratories in 2020 [29]. In this systematic review, our goal will be to examine the current extent of the application of genetic approaches to RABV surveillance globally and how, going forward, these approaches can contribute to the global strategy to eliminate human deaths from dog-mediated rabies.

Methods

Search strategy

A systematic search will be done on PubMed, Web of Science and Google Scholar electronic databases to identify original studies that reported genomic surveillance of rabies to support rabies elimination. Advanced searches with Boolean operators and quotations will be performed using the following key terms: ‘rabies AND (genom* OR sequenc* OR phylo* OR molecular) AND (control OR surveillance OR eliminat*)’. Further manual searches will be performed for additional relevant studies.

Selection of studies

Data will be extracted from studies of any design, including prospective and retrospective studies**.** A predefined set of inclusion and exclusion criteria will be applied to ensure the selection of relevant studies. The study selection process will follow the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and will be presented using a PRISMA flow diagram, detailing the number of studies screened, included, and excluded at each stage.

Inclusion and exclusion criteria

To ensure that relevant studies are included the following inclusion criteria will be used for screening: studies must address either canine rabies, human rabies or terrestrial wildlife rabies (i.e. not bat rabies), and use molecular techniques with sequencing data either for diagnosis or surveillance of rabies. We will exclude studies reported as literature reviews without presenting data, studies that are not published in English language, duplicated papers, that do not focus on rabies or include genomic/ sequencing data. We will follow PRISMA (Moher et al. 2009) guidelines to determine the Population, Intervention, Comparison and Outcome of the study (PICO), which for our study covers:

P (Population) = Rabies virus

I (Intervention) = genomic sequencing approaches

C (Comparison) = Known rabies control and prevention measures such as Mass dog vaccination and Post Exposure Prophylaxis (PEP)

O (Outcome) = Primary outcome - rabies control guidance; Secondary outcome – Other message from genomic surveillance

Management of identified articles and Quality assessment

All the articles identified from database searches will be exported for duplicate removal, screening of titles, abstracts and eligibility assessment according to the specified inclusion and exclusion criteria. Two independent reviewers will assess the quality of studies to be included in the systematic review. Any discrepancy observed between reviewers regarding the quality of selected study (s) will be resolved through discussion.

Data extraction and analysis

Data from eligible studies will be extracted into spreadsheets. with the following information , author and year of publication; location of study (country and subnational administrative unit if reported), study design, platform for sequencing, type of samples used (brain, saliva, vaccine or other), species of infected animal host (domestic dog, wildlife or other domestic animal, indicating the species involved), sample size (n), data analysis methods, conclusion(s) of the study and any control measure derived from the sequencing data.

The extracted data will be used to summarise the number of published papers and trends of time, the geographical coverage of the article the (richness of the genomic data available from different areas), what were the most commonly used sequencing platforms, and implications of the studies on how genomic data draws insight for rabies control and elimination.

Expected output

The systematic review will assess geographical coverage of rabies sequencing data in Africa, Asia, and Latin America, identifying gaps in genomic surveillance. It will provide recommendations on improving genomic surveillance through optimal sample types, sequencing platforms, and data management strategies. Additionally, key findings from included studies will highlight how genomic data provide critical insights to support rabies control efforts and contribute to the Zero by 30 goal.

Ethical approval and consent to participate

Not applicable

Consent for publication

The authors consented for publication

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29. Okeke IN, Ihekweazu C. The importance of molecular diagnostics for infectious diseases in low-resource settings. Nat Rev Microbiol. 2021;19: 547–548. doi:10.1038/s41579-021-00598-5

A.1.2 Example of the MeSH term used to select publications in PubMed database search

Search: **rabies AND genomic AND surveillance** Filters: **from 2000 - 2020**

("rabies"[MeSH Terms] OR "rabies"[All Fields]) AND ("genome"[MeSH Terms] OR "genome"[All Fields] OR "genomes"[All Fields] OR "genome s"[All Fields] OR "genomically"[All Fields] OR "genomics"[MeSH Terms] OR "genomics"[All Fields] OR "genomic"[All Fields]) AND ("epidemiology"[MeSH Subheading] OR "epidemiology"[All Fields] OR "surveillance"[All Fields] OR "epidemiology"[MeSH Terms] OR "surveillance"[All Fields] OR "surveillances"[All Fields] OR "surveilled"[All Fields] OR "surveillance"[All Fields])

Translations

rabies: "rabies"[MeSH Terms] OR "rabies"[All Fields]

genomic: "genome"[MeSH Terms] OR "genome"[All Fields] OR "genomes"[All Fields] OR "genome's"[All Fields] OR "genomically"[All Fields] OR "genomics"[MeSH Terms] OR "genomics"[All Fields] OR "genomic"[All Fields]

surveillance: "epidemiology"[Subheading] OR "epidemiology"[All Fields] OR "surveillance"[All Fields] OR "epidemiology"[MeSH Terms] OR "surveillance"[All Fields] OR "surveillances"[All Fields] OR "surveilled"[All Fields] OR "surveillance"[All Fields]

Appendix B:

Chapter 3 Appendix

B.1 Supplementary Files

B.1.1 Protocol

The study was approved by the Medical Research Coordinating Committee of the National Institute for Medical Research (NIMR/HQ/R.8a/vol.IX/2788), the Ministry of Regional Administration and Local Government (AB.81/288/01), and Ifakara Health Institute Institutional Review Board (IHI/IRB/No:22-2014) in Tanzania; the University of Nairobi Institute of Tropical and Infectious Diseases (P947/11/2019) and the Kenya Medical Research Institute (KEMRI-SERU protocol No. 3268) in Kenya; and the Research Institute for Tropical Medicine (RITM), Department of Health (2019-023) in the Philippines. Sequencing of samples originating from Nigeria was undertaken on archived diagnostic material collected as a part of national surveillance.

NOTE: Steps 1–4 are prerequisites, Steps 5–16 describe the sample-to-sequence-to-interpretation workflow for RABV nanopore sequencing (Figure **3.1**). For subsequent steps in the protocol that need pulse centrifugation, do 5-15 seconds at 10-15000 g.

B.1.1.1 Computational environmental setup

1.1. Open the Oxford Nanopore Technology (ONT) website (Oxford Nanopore Technology, 2023a) and create an account to access nanopore-specific resources.

1.1.1. Log in and install ONT sequencing and basecalling software (Oxford Nanopore Technology, 2023b).

1.2. Open GitHub (GitHub, 2023) and create an account.

1.2.1. Go to the artic-rabv (Brunker, 2020/2022) and MADDOG repositories (Campbell, 2021/2022) and follow the installation instructions.

B.1.1.2 Design or update the multiplex primer scheme

NOTE: Existing RABV schemes are available in the Artic-rabv repository (Brunker, 2020/2022). When targeting a new geographic area, a new scheme should be designed, or an existing scheme modified to incorporate additional diversity.

2.1. Choose a genome reference set to represent the diversity in the study area - this is typically a set of publicly available sequences (e.g., from NCBI GenBank) or preliminary in-house data. Follow steps 2.1.1–2.1.4 to use RABV-GLUE (Centre for Virus Research, 2023), a RABV sequence data resource, to filter and download NCBI sequences and associated metadata.

NOTE: Choose reference sequences with complete genomes i.e., without gaps and masked bases. Choosing up to 10 sequences that are distinct as a reference set for primer design is recommended. If the available sequence data is incomplete or not representative of the study area, refer to the advice (Davis & Jorgensen, 2022; Döring & Pfeifer, 2023; Itokawa et al., 2020) in 0

2.1.1. Navigate to the NCBI RABV Sequences by Clade page from the Sequence Data drop-down menu on RABV-GLUE. Click the Rabies Virus (RABV) link to access all available data or select a particular clade of interest. Use the filter option to Add filters that fit the desired criteria e.g., country of origin, sequence length. Download sequences and metadata.

2.2. Generate a primer scheme for multiplex PCR following the instructions provided by Primal Scheme (Quick et al., 2017). A 400 bp scheme with a 50 bp overlap is recommended to sequence low quality samples. Download and save all outputs (do not edit the file or primer names).

NOTE: The scheme will be indexed to the first sequence in the input fasta, henceforth referred to as the ‘index reference’ (Figure B. 1). B.1.2 for options to optimize primer performance.

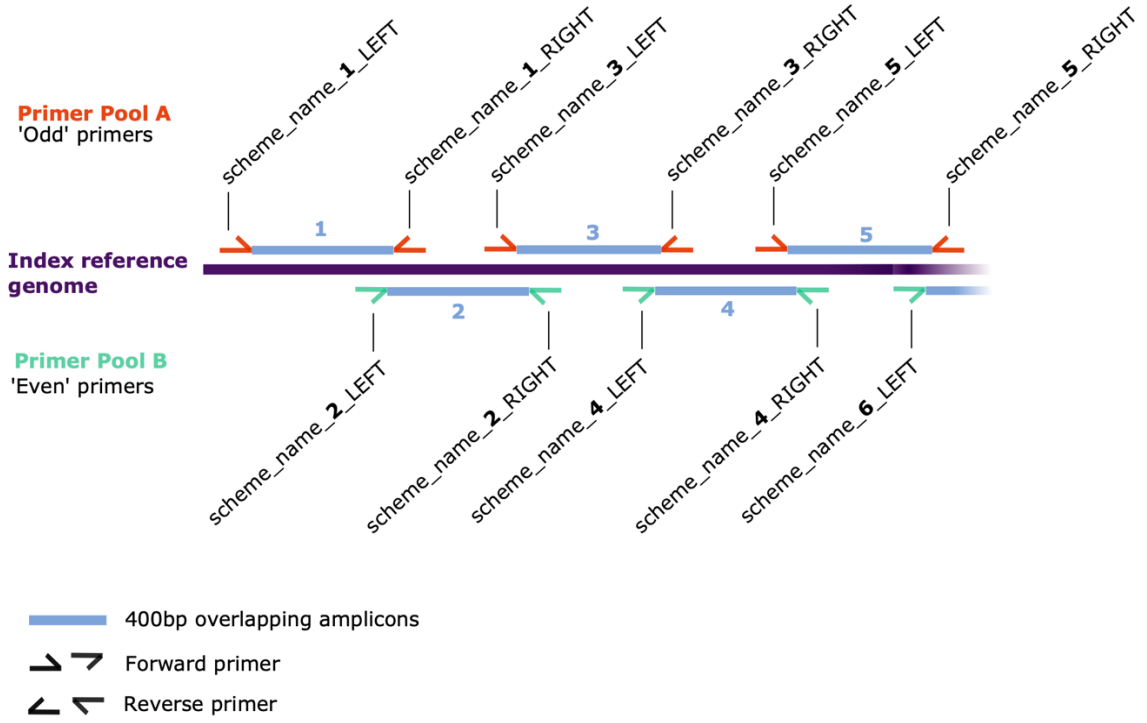


Figure B. 1. Primer scheme schematic. Shows annealing positions along the 'Index reference genome' (dark purple) for pairs of forward and reverse primers (half arrows) which are assigned in two separate pools: A (red) and B (green). Primer pairs generate 400 bp overlapping amplicons (blue) which are numbered sequentially along the index reference genome in the format 'scheme_name_X_DIRECTION' where 'X' is a number referring to the amplicon generated by the primer, and 'DIRECTION' is either 'LEFT' or 'RIGHT' describing the forward or reverse respectively. Odd or even value of 'X' determines the Pool A/B.

B.1.1.3 Set up RAMPART and ARTIC bioinformatics pipeline

3.1. Refer to B.1.3 to set up a directory structure to manage input/output files for RAMPART and the ARTIC bioinformatics pipeline.

B.1.1.4 Biosafety and laboratory setup

4.1. Handle potentially rabies positive samples in Biosafety Level (BSL) 2 or 3 conditions.

4.2. Ensure laboratory staff have completed rabies pre-exposure vaccination and undergo monitoring of immunity according to WHO recommendations (WHO, 2017a).

4.3. Ensure dedicated standard operating procedures and risk assessments, following national or international guidelines are in place for the laboratory.

4.4. Required lab set-up: Minimize contamination by maintaining physical separation between pre- and post-PCR areas. In laboratories with limited space or in field lab settings use portable glove boxes or makeshift lab stations to minimize contamination.

4.5. In this protocol ensure designating separate areas for:

4.5.1. Sample extraction: Set up a BSL2/3 cabinet/glove box to handle biological material and perform inactivation and RNA extraction.

4.5.2. Template area: Set up a BSL1 cabinet/glove box for the addition of template (RNA/cDNA) to pre-prepared reaction master mix.

4.5.3. Master mix area: Set up a designated clean area (BSL1 cabinet/glove box) for preparation of reagent master mixes. No template in this area.

4.5.4. Post-PCR area: Set up a separate area for work on amplicons and sequencing library preparation.

NOTE: All areas should be cleaned with a surface decontaminant and UV sterilized before and after use.

B.1.1.5 Field sample collection and diagnosis

NOTE: Samples must be collected by trained and immunized personnel wearing personal protective equipment and following the referenced standard procedures (Lembo & Prevention, 2012; ‘Terrestrial Manual Online Access’, 2023; World Health Organization et al., 2018).

5.1. Collect sample via the foramen magnum i.e., the occipital route, as described in detail in Mauti et al. 2020 (Mauti et al., 2020).

5.2. Diagnose rabies in the field with rapid diagnostic tests and confirm in the laboratory using recommended procedures47 such as the Direct Fluorescent Antibody Test (DFA), the Direct Rapid Immunohistochemical Test (DRIT) (Lembo et al., 2006; Patrick et al., 2019) or the real time RT-PCR (Marston et al., 2019).

5.3. Use confirmed positive brain samples for RNA extraction or store in the freezer at -20 °C for 2–3 months and -80 °C for longer periods. Preserve RNA for storage and transport using a suitable DNA/RNA stabilization medium.

B.1.1.6 Sample preparation and RNA extraction

NOTE: Use a spin-column based viral RNA extraction kit suitable for the sample type.

6.1. Using a wooden applicator remove a ~3 mm cube of brain tissue (from fresh, thawed or RNA-stabilized sample). 3 mm is about the height of a font 12 in print.

6.2. Label a pre-filled reinforced 2 mL tube with 1.4 mm ceramic beads or prepare a tube manually by adding a quantity of beads approximate to one fill of a 200 µL PCR tube.

6.3. Disrupt the tissue in the RNA extraction kit's lysis buffer, using manufacturer's recommended volume, until fully homogenized. Allow foam to settle and homogenize again if required.

NOTE: Use closed tube bead-based homogenization to limit sample exposure. If not possible, use other suitable mechanical disruptors (e.g., rotor-based) or a manual micro pestle. However, these may be less effective than bead beating on hard to disrupt tissue (tissue samples may harden in certain storage media).

6.4. Centrifuge the lysate as per the manufacturer's instruction and use a pipette to transfer the supernatant to a new microcentrifuge tube. Use only this supernatant in subsequent steps.

6.5. Follow the RNA extraction kit's spin column instructions to obtain purified RNA.

6.6. Include a negative extraction control (NEC) here and take all the way through to the sequencing stage.

B.1.1.7 cDNA preparation

7.1. In the master mix area, prepare a master mix for first strand cDNA synthesis according to the number of samples and controls to be processed (with an excess volume of 10% to ensure adequate reagent; Table B. 1). A no template control (NTC) should be included at this stage.

Table B. 1. Master mix and thermal cycler conditions for cDNA preparation

Master mix for cDNA preparation	
Component	**Volume per reaction (µL)**
First strand cDNA synthesis master mix	2
Nuclease free water (NFW)	3
Thermal cycler conditions for cDNA preparation	
Temp	**Time**
25 °C	2 min
55 °C	10 min
95 °C	1 min
4 °C	hold until next step

7.2. Label 0.2 ml PCR strip tubes and aliquot 5 µL of the master mix into tubes.

7.3. Take the prepared tubes to the Template area. Add 5 µL of RNA into each labelled tube, including the NEC. Add 5 µL of Nuclease free water (NFW) to the NTC.

7.4. Incubate in a thermal cycler following the conditions mentioned in Table B. 1

NOTE: Optional Pause Point: cDNA can be stored at -20°C for up to a month, if necessary, but proceeding to PCR is preferred.

B.1.1.8 Primer pool stock preparation

NOTE: This step is only necessary if making new stocks from individual primers, after which pre-prepared stock solutions can be used.

8.1. Prepare a primer pool of 100 µM stock in the master mix area.

8.2. Resuspend the lyophilized primers in 1x TE or NFW at a concentration of 100 µM each. Vortex thoroughly and spin down.

NOTE: In the following steps individual primers are separated into two primer pools; odd numbered (named Pool A) and even numbered (named Pool B) to avoid interactions between primers flanking amplicon overlaps. These pools of primers generate overlapping 400bp amplicons spanning the target genome.

8.3. Arrange all odd numbered primers in a tube rack. Generate a primer pool stock by adding 5 µL from each primer to a 1.5 mL microcentrifuge tube labeled "primer scheme name - Pool A (100 µM)".

8.4. Repeat the process for all even numbered primers and label "primer scheme name - Pool B (100 µM)".

8.5. Dilute each primer pool 1:10 in molecular grade water, to generate 10 µM primer stocks.

NOTE: Make multiple aliquots of 10 µM primer dilutions and freeze them in case of degradation or contamination.

B.1.1.9 Multiplex PCR

9.1. Prepare two PCR master mixes, one for each of primer Pool A and primer Pool B in the master mix area.

9.1.1. Use a final concentration of 0.015 µM per primer. Calculate the required primer pool volume for the PCR reaction (Table B. 2) using the following formula:

Primer pool volume = Number of primers x Reaction volume x 0.015/ Concentration (µM) of primer stock

Table B. 2. Master mix and thermal cycler conditions for multiplex PCR

Master mix for multiplex PCR			
Component	**(Pool A master mix)**	**(Pool B master mix)**	
	Volume per reaction (µL)	**Volume per reaction (µL)**	
PCR Master Mix	6.25	6.25	
Primer Pool A or Pool B (10 µM)	*Calculated in step 5.1.1*	*Calculated in step 5.1.1*	
Nuclease-free water	up to 10 µL total volume	up to 10 µL total volume	
TOTAL	10	10	
Thermal cycler conditions for multiplex PCR			
Stage	**Temp**	**Time**	**Cycles**
Initial denaturation	98 °C	30 s	1
Denaturation	98 °C	15 s	35*
Annealing and extension	65 °C	5 min	
Hold	4 °C	hold	1

B.1.1.10 PCR clean up and quantification

10.1. Perform all work from this point on in the post-PCR area.

10.2. Aliquot solid-phase reversible immobilization (SPRI) beads into microcentrifuge tubes from the main bottle. Store at 4 °C.

10.3. Warm a SPRI bead aliquot to room temperature (RT; ~20 °C) and thoroughly vortex until beads are fully resuspended in solution.

10.4. In 1.5 mL tubes, combine primer pool A and primer pool B PCR products for each sample. If necessary, add water to bring the volume to 25 µL.

10.5. Add 25 µL of SPRI beads to each sample (1:1 bead:sample ratio). Mix by pipetting up and down or gently tap tube.

10.6. Incubate at RT for 10 min, occasionally inverting or flicking tubes.

10.7. Place on a magnetic rack until the beads and solution have fully separated. Remove and discard supernatant, taking care not to disturb the bead pellet.

10.8. Wash twice with 80% ethanol (warmed to RT).

10.8.1. Add 200 µL of ethanol to the pellet. Wait for 30 s to ensure the beads are washed properly.

10.8.2. Carefully remove as much ethanol as possible using a 10 µL tip.

10.8.3. Repeat steps 10.8.1–10.8.2 to wash pellet for a second time.

10.9. Remove all traces of ethanol. Air dry until trace ethanol has evaporated (~1 min), when this happens the pellet should go from shiny to matt. Take care not to overdry (if pellet is cracking it is too dry) as this will affect DNA recovery.

10.10. Resuspend the beads in 15 µL of NFW and incubate at RT (off magnetic rack) for 10 min.

10.11. Return to the magnetic rack and transfer the supernatant (cleaned product) to a fresh 1.5 mL tube.

10.12. Prepare a 1:10 dilution of each sample (2 µL of product +18 µL of NFW).

NOTE: Be very careful at this stage to avoid cross-contamination. Only have one amplicon tube open at a time. Aliquot 18 µL of water into the tubes first (in clean master mix area).

10.13. Measure the DNA concentration of each diluted sample using a highly sensitive and specific fluorometer as described in protocols.io (Brunker, 2020; Quick, 2020).

B.1.1.11 Normalization

11.1. Use the normalization template (B.1.4) and DNA concentration (ng/µL) of each sample to calculate the volume of diluted (or neat) sample required for 200 fmol of each sample in a total volume of 5 µL.

11.2. Label new PCR tubes and add computed volumes of NFW and sample to obtain normalized DNA.

11.3. Use the computed volume for undiluted (neat) samples if over 5 µL of the diluted sample is required to obtain 200 fmol.

NOTE: Optional Pause Point: At this point the cleaned-up PCR product can be stored at 4 °C for up to a week or placed at -20°C for longer-term storage if needed

B.1.1.12 End-prep and barcoding

NOTE: The next steps assume use of specific reagents from nanopore-specific barcoding and ligation sequencing kits, please refer to the Materials list in (B.1.5) for details. The protocol is transferable across different chemistry versions, but user should take care to use compatible kits according to the manufacturer's information.

12.1. End repair and dA-tailing

12.1.1. Set up the end-prep reaction for each sample mentioned in

Table B. 3. Prepare a master mix according to the number of samples (plus 10% excess). Take care when pipetting as reagents are viscous.

Table B. 3. Master mix and thermal cycler conditions for end prep reaction

Master mix for End-prep reaction	
Component	**Volume per reaction (µL)**
NFW	3.5
End Prep Reaction buffer	1
End Prep enzyme mix	0.5
TOTAL	5
Thermal cycler conditions for End-prep reaction	
Temp	**Time**
20°C	15 min
65°C	15 min
4°C	1 min

12.1.2. Add 5 µl of master mix into each tube of normalized DNA (5 µl). Total reaction mix should be 10 µl. Change tips each time and only have one tube open at a time.

12.1.3. Incubate in a thermal cycler under the conditions mentioned in

Table B. 3.

12.2. Barcoding

12.2.1. Aliquot the barcodes from the barcoding kit to PCR strip tubes at 1.25 µL/tube, record barcode assigned to each sample.

12.2.2. Add 0.75 µL of the end prepped sample to its assigned barcode aliquot.

12.2.3. Set up the ligation reaction for each sample as follows. Prepare a master mix according to the number of samples (plus 10% excess) (Table B. 4)

Table B. 4. Master mix and thermal cycler conditions for barcoding.

Master mix for Barcoding	
Component	**Volume per reaction (µL)**
NFW	3
Ligation Master Mix	5
TOTAL	8

Thermal cycler conditions for Barcoding	
Temperature	**Time**
20 °C	20 min
65 °C	10 min
4 °C	1 min

12.2.4. Add 8 µl of ligation master mix to end-prepped sample + barcodes, giving a total reaction of 10 µl.

12.2.5. Incubate in a thermal cycler using the conditions mentioned in Table B. 4.

12.3. SPRI bead clean-up and DNA quantification

12.3.1. Thaw Short Fragment Buffer (SFB) at RT, mix by vortexing, pulse centrifuge, and place on ice.

12.3.2. Pool all barcoded samples together in a 1.5 mL lobind microcentrifuge tube. So as not to make the clean-up volume too large to use: 12–24 samples (10 µL), up to 48 samples (5 µL), up to 96 samples (2.5 µL) from each native barcoding reaction.

12.3.3. Add 0.4x volume of SPRI beads to barcoded pool. Mix gently (flicking or pipetting) and incubate at RT for 5 min.

12.3.4. Place the samples on magnet until beads have pelleted and supernatant is completely clear (~2 min). Remove and discard the supernatant. Take care not to disturb the beads.

12.3.5. Wash twice with 250 µL of SFB.

12.3.6. Remove the tube from magnet and resuspend the pellet in 250 µL of SFB. Incubate for 30 s, pulse centrifuge and return to magnet. Remove supernatant and discard.

12.3.7. Repeat step 12.3.6 to perform a second SFB wash.

12.3.8. Pulse centrifuge and remove any residual SFB.

12.3.9. Add 200 µL of 80% (RT) ethanol to bathe the pellet. Remove and discard ethanol being careful not to disturb the bead pellet. Air dry for 30 s or until the pellet has lost its shine.

12.3.10. Resuspend in 22 µL of NFW at RT for 10 min.

12.3.11. Place on magnet, leave to settle for ~2 min, then carefully remove solution and transfer to a clean 1.5 mL microcentrifuge tube.

12.3.12. Use 1 μL to obtain DNA concentration as described previously (section 10.13).

NOTE: Optional Pause Point: At this point the library can be stored at 4°C for up to a week or -20°C for longer-term storage, but it is preferable to continue with adapter ligation and sequencing.

B.1.1.13 Sequencing

13.1. Prepare computer (refer also to Prerequisites sections 1–4)

13.1.1. Check there is enough space to store new data (min 150 GB); that data from old runs is backed up/moved to server before deleting and that the latest version of MinKNOW is installed.

13.2. Remove the stored flow cell from the fridge and allow to reach RT.

13.3. Adapter ligation (1 h)

13.3.1. Pulse centrifuges the adapter mix and ligase and place on ice

13.3.2. Thaw elution buffer (EB), SFB and ligation buffer at RT. Mix by vortexing, pulse centrifuge, place on ice

13.3.3. Prepare the adapter ligation master mix below (Table B. 5), combining reagents in specified order in a low bind tube:

NOTE: Alternatives for adapter ligation master mix reagents (Table B. 5) can be used depending on availability at the lab. See B.1.4 and B.1.5 for list of alternatives.

Table B. 5. Adapter ligation master mix and alternative adapter ligation reagents

Adapter quick ligation module master mix	
Component	**Volume per reaction (μL)**
Barcoded amplicon pool	20*
NFW (to make up to 20 μL)	0*
Adapter Mix	5
Adapter ligation buffer (5x)	10

Adapter ligation enzyme	5
TOTAL	40
* 100–200 fmol of final library is optimal	
Ligation Kit Option 1	
Adapter TA ligation master mix	
Component	**Volume per reaction (µL)**
Barcoded amplicon pool	20*
NFW (to make up to 20 µL)	0*
Adapter Mix	5
Blunt/TA Ligase master mix	25
TOTAL	50
* 100–200 fmol of final library is optimal	
Ligation Kit Option 2	
Adapter ligation module master mix	
Component	**Volume per reaction (µL)**
Barcoded amplicon pool	20*
NFW (to make up to 20 µL)	0*
Adapter Mix	5
Adapter ligation master mix	25
Adapter ligation enhancer	0.5
TOTAL	50.5
* 100–200 fmol of final library is optimal	

Use computation in B.1.4 worksheet to get the volume of DNA library equivalent to 200 fmol. If less than 20 µL is computed, add NFW to make up to 20 µL.

13.3.4. Mix by gentle flicking and pulse centrifuge. Incubate at RT for 20 min.

NOTE: During incubation start preparing the flow cell (section 13.5).

13.4. Clean up using SPRI beads (do not use ethanol as in earlier cleanups)

13.4.1. Add 0.4x volume of SPRI beads (RT) to the samples. Incubate at RT for 10 min, gently flick and invert intermittently to aid mixing.

13.4.2. Place on magnet until beads and solution have fully separated (~5 min). Remove and discard the supernatant; take care not to disturb the bead pellet.

13.4.3. Wash twice with 125 µL of SFB.

13.4.4. Resuspend the pellet completely with 125 μL of SFB by pipette mixing. Leave to incubate for 30 s.

13.4.5. Pulse centrifuge to collect liquid at tube base and place on magnet. Remove the supernatant and discard.

13.4.6. Repeat step 13.4.4 - 13.4.5 to wash pellet for a second time.

13.4.7. Pulse centrifuge and remove the excess SFB.

13.4.8. Resuspend in 15 μL of EB and incubate for 10 min at RT.

13.4.9. Return to magnet for ~2 min and then carefully transfer solution to a clean 1.5 mL microcentrifuge tube.

13.4.10. Quantify 1 μL of the eluted library as described previously on step 10.13.

NOTE: For best results proceed directly to MinION sequencing but the final library can be stored in EB at 4 °C for up to a week if needed.

13.5. Run a flow cell quality check.

13.5.1. Connect the sequencing device to laptop and open the sequencing software.

13.5.2. Select flow cell type and click Check Flow Cell then Start Test

13.5.3. Once complete, the total number of active (i.e., viable) pores will be displayed. A new flow cell should have >800 active pores, if it does not contact manufacturer for a replacement.

13.6. Priming and loading the flow cell (20min)

13.6.1. Thaw the following reagents at room temp then place on ice: sequencing buffer, flush tether, flush buffer, loading beads

13.6.2. Vortex the sequencing buffer and flush buffer, pulse centrifuge, and place on ice

13.6.3. Pulse centrifuge flush tether and mix by pipetting, place on ice.

13.6.4. Prepare the flow cell priming mix by adding 30 µL of flush tether directly to the tube of flush buffer from a flow cell priming kit and mix by pipetting.

13.6.5. Mix the loading beads by pipetting immediately prior to use as they settle quickly.

13.6.6. In a fresh tube prepare the final library dilution for sequencing as mentioned in Table B. 6.

Table B. 6. Library mix for sequencing

Final library dilution	
Library mix reagents	**Volume per reaction (µL)**
Sequencing Buffer	37.5
Loading Beads	25.5
DNA library	12*
Elution Buffer (to make up DNA library volume)	0*
TOTAL	75
* 5-50 fmol of final library is optimal.	

NOTE: Use computation in B.1.4 worksheet to get the volume of DNA library equivalent to 50 fmol. If less than 12 ul is computed, add elution buffer to make up to 12 ul.

13.6.7. Flip back the sequencing device lid and slide the priming port cover clockwise so that priming port is visible (Figure B. 2)

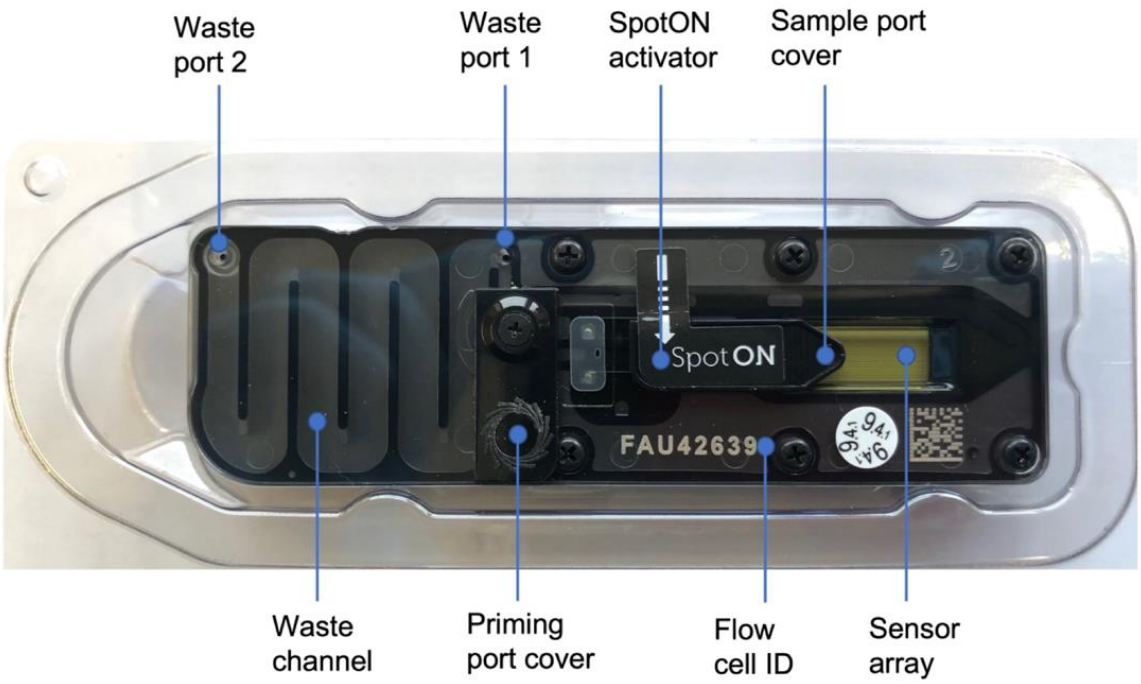


Figure B. 2. Nanopore flow cell. Blue labels illustrate the different parts of the flow cell, including the priming port cover which covers the priming port where the priming solution is added, the SpotON sample port cover covering the sample port where the sample is added in a dropwise fashion, the waste ports 1 and 2, and the flow cell ID.

13.6.8. Remove air bubbles carefully by setting a P1000 to 200 µL, insert the tip into the priming port and turn the wheel until you can see a small volume entering the pipette tip (max turn to 230 µL)

13.6.9. Load 800 µL of flow cell priming mix into the flow cell via the priming port taking care to avoid bubbles.

13.6.10. Leave for 5 min.

13.6.11. Lift the sample port cover gently and load 200 µL of priming mix into flow cell via Priming port using a P1000 pipette

13.6.12. Pipette mix library mix prior to loading ensuring loading beads in the master mix are resuspended before loading.

13.6.13. Load 75 µL of library mix to flow cell via the sample port in a dropwise fashion. Ensure that each drip flows into the port before adding the next.

13.6.14. Replace the sample port cover gently making sure bung enters sample port.

13.6.15. Close the priming port and replace the sequencing device lid.

13.7. Sequencing run (48 h maximum)

13.7.1. Connect the sequencing device to the laptop and open sequencing software.

13.7.2. Click start and then click Start Sequencing.

13.7.3. Click New Experiment and follow the sequencing software GUI workflow to set up the parameters for the run.

13.7.4. Type in the Experiment name, Sample ID (e.g., rabv_run1) and choose the Flow Cell Type from the drop-down menu.

13.7.5. Continue to kit selection and choose the relevant ligation sequencing kit and native barcoding kit(s) used.

13.7.6. Continue to Run options. Keep defaults unless you want the run to stop automatically after a certain number of hours (runs can be stopped manually at any time).

13.7.7. Continue to Basecalling. Choose to turn Basecalling On or Off according to the computing resources (see computer setup). Choose Edit Options under barcoding and ensure Barcode Both Ends is turned on. Save and continue to Output section.

13.7.8. Accept defaults and continue to final review, check settings and record details in worksheet (B.1.4). Click Start.

NOTE: If flow cell is being reused adjust the starting voltage (in the advanced section of the run options) as indicated by the scheme in B.1.4.

13.7.9. Record the initial active channels - if this is significantly lower than the QC check then restart the sequencing software, if still lower reboot the computer.

13.7.10. Record the initial channels in strand vs single pore to give an approximate pore occupancy. This number will fluctuate so give an approximation.

13.7.11. Monitor the run as it progresses.

B.1.1.14 Live and offline basecalling

NOTE: These instructions assume the pre-existing directory structure provided in the artic-rabv repository, and that the Prerequisites Step 1 and 3 of the Protocol have been followed.

14.1. In the artic-rabv/analysis folder, navigate to the relevant project directory and create a new directory for the run using the Sample ID provided to MinKNOW as the run_name.

cd path/artic-rabv/analysis/project_name

mkdir -p run_name/

14.2. Live basecalling

NOTE: To perform Nanopore basecalling in real-time, laptops require a NVIDIA CUDA-compatible GPU. Ensure instructions for GPU basecalling setup have been performed using the guppy protocol (Nanopore Community, 2023b)

14.2.1. During run setup, turn live basecalling on.

14.2.2. Use RAMPART to monitor the sequencing coverage in real time as per instruction below.

14.2.3. In the computer's terminal, activate the artic-rabv conda environment:

conda activate artic-rabv

14.2.4. Create a new directory for the rampart output inside the run_name directory and navigate into it:

cd /path/artic-rabv/analysis/project_name/run_name

mkdir rampart_output

cd rampart_output

14.2.5. Create a barcodes.csv file to pair barcodes and sample names. It should have one line per barcode and only specify barcodes that are present in your library, with the headings “barcode” and “sample”. Follow the example in the artic-rabv directory:

artic-rabv/analysis/example_project/example_run/rampart_output/barcodes.csv

14.2.6. Start RAMPART by providing the relevant protocol folder and path to the fastq_pass folder in the MinKNOW output for the run:

rampart --protocol /path/artic-rabv/rampart/scheme_name_V1_protocol –basecalledPath <insertpathTo Fastqpassfolder>

14.2.7. Open a browser window and navigate to localhost:3000 in the URL box. Wait for sufficient data to be basecalled before results appear on the screen.

14.3. Offline basecalling (performed post-run)

14.3.1. If live basecalling was not set, the output from MinKNOW will be raw signal data (fast5 files). You will not be able to use RAMPART during the run. Fast5 files can be converted to basecalled data (fastq files) post-run using Guppy (see setup in Prerequisites 1.1.1.). You can run RAMPART post-hoc on basecalled data.

14.3.2. Run the guppy basecaller:

guppy_basecaller -c dna_r9.4.1_450bps_fast.cfg -i /path/to/reads/fast5_* -s /path/artic-rabv/analysis/project_name/run_name -x auto -r

-c is the config file to specify the basecalling model, -i is the input path, -s is the save path,

-x specifies basecalling by GPU device (exclude if using CPU version of Guppy), -r specifies to search input files recursively. fast5_* will search both the fast5_pass and fast5_fail folders.

NOTE: the config file (.cfg) can be changed to a High Accuracy basecaller by replacing _fast with _hac. This will take significantly longer.

B.1.1.15 Washing the flow cells

15.1. Flow cells can be washed and reused to sequence new libraries if pores are still viable. See instructions for washing at ONT flow cell wash protocol (Nanopore Community, 2023a).

B.1.1.16 Analysis and interpretation

16.1. Consensus sequence generation with ARTIC bioinformatics pipeline.

16.1.1. Follow the instructions detailed in the artic-rabv GitHub repository (Brunker, 2020/2022) in the rabv_protocols folder to generate consensus sequences from raw fast5 or basecalled fastq files.

NOTE: Refer to Artic pipeline - Core pipeline (Artic Pipeline, 2023) for further guidance.

16.2. Optional: Analyze the average read depth per amplicon.

16.2.1. Adapt the scripts available from the artic-rabv repository, referring to Supplemental File 1. Briefly, depth statistics are generated using SAMtools (Li et al., 2009) and coverage per nucleotide plotted in R.

16.3. Phylogenetic analysis using GLUE.

16.3.1. From RABV_GLUE (Centre for Virus Research, 2023) select Analysis > Genotyping and Interpretation tab, and Add Files, selecting your fasta file of consensus sequences.

16.3.2. Click Submit and wait. Once analyses are complete, the Show Analysis button will be available to click, showing clade and subclade assignments; coverage per gene; variation from reference sequences; closest relative.

16.3.3. Relevant contextual sequences can also be identified in the Sequence Data > NCBI Sequences by Clade section.

16.3.4. Select the clade identified or click Rabies Virus (RABV) to see all available sequences.

16.3.5. Filter for relevant sequences (e.g., country of origin)

16.3.6. Download these sequences and corresponding metadata for analysis and comparison.

16.4. Lineage assignment using MADDOG (Campbell, 2021/2022).

16.4.1. Pull the MADDOG repository from GitHub to ensure you are working with the most up to date version.

16.4.2. Create a folder within your local MADDOG repository (previously created at the Prerequisites section), called the run name.

16.4.3. Inside the folder, add the fasta file containing your consensus sequences.

16.4.4. Add a metadata file to the folder. This file must be a csv with 4 columns called ‘ID’, ‘country’, ‘year’ and ‘assignment’, detailing the sequence IDs, the country of sampling, and year of sample collection, while the ‘assignment’ column should be blank.

NOTE: The ID in the metadata file must exactly match the IDs in the fasta file.

16.4.5. In the command line interface, activate the conda environment: conda activate MADDOG.

16.4.6. In the command line interface, navigate to the MADDOG repository folder.

16.4.7. Initially, lineage assignment is undertaken on sequences to check for any potential abnormalities, and to identify if running the longer lineage designation step would be appropriate: sh assignment.sh

16.4.8. When prompted, enter Y to indicate you have pulled the repository and are working with the most up to date version of MADDOG

16.4.9. When prompted, enter the name of the folder within the MADDOG repository folder that contains your fasta file.

16.4.10. When lineage assignment is complete, check the output file in your folder. If the output is as expected, and there are multiple sequences assigning to the same lineage, lineage designation should then be run.

16.4.11. If running lineage designation, delete the assignment output file just created.

16.4.12. In the terminal, inside the MADDOG repository folder, run the command: sh designation.sh

16.4.13. When prompted, enter Y to indicate you have pulled the repository and are working with the most up to date version of MADDOG

16.4.14. When prompted, enter the folder name within the MADDOG repository folder containing your fasta file and metadata. This will output lineage information about each sequence, a phylogeny of the new and relevant previous sequences (from 16.3.6), and hierarchical information about the lineages.

16.4.14.1 When the initial analysis has been completed, you will be asked if you want to also test for emerging and undersampled lineages. If this is required, enter Y when prompted. Otherwise, enter N.

16.1.14.2 If new lineages are found, you will be prompted to have these lineages confirmed. To do this, enter Y when prompted and follow the instructions in the resultant NEXT_STEPS.eml file. Otherwise, enter N.

16.4.15. Full details of the protocol, usage, and outputs in Campbell et al. 2022 (Campbell et al., 2022).

B.1.2 Primer scheme design and optimization and amplicon read depth analysis

NOTE: This Supplementary File contains further advice on how to design, optimize and assess the performance of a primer scheme. This is for guidance only. It assumes a level of familiarity with bioinformatic tools and/or programming experience. If you are not comfortable with these steps, input from an experienced user is highly recommended.

Address incomplete or non-representative reference data for the input to Primal Scheme.

NOTE: If the reference data which are available are incomplete or not representative of your study area, there may be gaps in your sequencing coverage due to 'amplicon drop-out' (failure of the primers to bind and amplify the genome in this region). This is more likely to occur if there is limited existing knowledge of the diversity of circulating lineages, or if you are sequencing viruses sampled across a wide geographic range.

Choose publicly available data

In the absence of publicly available data, ideally some preliminary metagenomic sequencing should be performed to obtain 1 or more reference sequences.

If initial metagenomics is not possible, always try to choose the most complete genomes available from the area.

You can choose genomes from a different geographical area as long as it is identified as closely related lineages to the ones circulating in your study area.

Incorporate partial genomes obtained from GenBank. Gap-fill sequencing can be concatenated and spliced into the reference genome(s). Take care to ensure each part of the genome is correctly aligned and not to overrepresent any particular partial sequence.

NOTE: The majority of publicly available RABV sequences are partial genome data, particularly for nucleoprotein and glycoprotein genes. Therefore, there is potentially a lot to gain by including existing partial genome information in primer design. This is most useful to fill in missing ends of otherwise representative genomes, since the ends are generally

highly conserved, or if a particular region is failing to sequence for certain lineage(s). Similarly, targeted gap-fill sequencing (across problematic regions of amplicon drop-out) can improve reference sequence data when redesigning primers in problematic genome regions (at relatively low cost) to allow primer redesign in these areas. (Refer to part 2).

Use Primal Scheme to generate a new primer scheme.

NOTE: If you are unsure about these steps, you may instead modify individual primer sequences according to section 3 below.

Sequence across large internal gaps using targeted PCRs.

Targeted singleplex PCRs attempt to sequence across regions of amplicon drop-out by generating amplicons larger than 400bp to span across the gap. The partial genomes (large amplicons) generated can be used in Step 1.1.4. above.

NOTE: Before beginning this step, you may seek bioinformatics help to check if there is already any low-level representation of larger 'gap-filling' amplicons in your existing sequence data, where primers flanking the problematic region have naturally paired and amplified across it.

Identify the closest forward and reverse primers flanking the problematic region that generated amplicons with good read depth in previous rounds of sequencing.

NOTE: If the gap is at the end of the sequence, try using a universal rabies primer (since sequence ends are highly conserved).

Select a PCR polymerase which is optimized for amplifying the size of amplicon you are attempting (a standard polymerase effectively amplifies fragments up to approximately 5kb).

NOTE: Generation of larger amplicons relies on greater sample integrity.

Carry out singleplex PCR according to the manufacturer's instructions and annealing temperature of the selected primers.

Check for presence of the gap-fill amplicon product on an agarose gel.

If present, process the gap-fill amplicon for sequencing following the Workflow Step 5 onwards.

NOTE: Molarity calculations will vary for different amplicon lengths, adjust accordingly. Multiple gap-fill amplicons should be normalized in equimolar quantities to each other and any other samples being processed in parallel.

Individual primer modification and amplicon depth equalization.

Consistent amplicon dropout or low average read depth (refer to section 5 below) may result from *(a)* poor annealing of one or both of the primers (from the pair) to the genome variant, or *(b)* interaction between primers (in the scheme) resulting in primer sequestration as mentioned by Itokawa et. al, 2020. The sequence and/or final concentration of individual primers may be optimized to equalize any large differences in average read depth per amplicon. Equalizing average read depth between amplicons helps to achieve full genome coverage earlier in the run, promoting re-use of the flow cell and cost-saving.

NOTE: If there are a large number of amplicons with low average read depth, or if new lineage diversity has emerged during your initial sequencing, it is recommended to generate a new primer scheme in Primal Scheme using an updated input fasta which includes your new sequences or partial genomes (refer to section 1).

Optimizing individual primer sequences.

NOTE: Primer sequences may be modified with nucleotide substitutions either within the original primer if appropriate, or by creating an alternative primer to represent a subset of the circulating diversity. Or alternatively, the position of the whole primer can be shifted to an area which is better conserved among the diversity of circulating lineages.

Create an alignment of available sequences to the ‘index reference’ genome of the whole primer scheme.

NOTE: These may include whole and/or partial genomes from GenBank, your own sequencing or gap-fill sequences (refer to section 2).

For each amplicon with low depth of coverage, visually inspect annealing sites of both forward and reverse primers and compare mismatching of the primers to the various genomes in the alignment.

NOTE: An appropriate genome alignment, manipulation or visualization software may be used in place of visual inspection if comfortable with this computationally.

Determine whether one or both of the primers should be redesigned and how (e.g nucleotide substitution, alternative primer, shift position of primer). ***NOTE:*** If both primers appear not to have significant mismatches, low read depth could be due to hybridization and sequestration by another primer in the scheme. If this is the case, consider increasing the concentration of both primers in the pair. Primer-primer interactions can be checked with a primers analyzer tool. If changing the position of a primer, make sure the new position is known with respect to the index reference genome.

Try to avoid introducing new primer-primer interactions to the scheme with manually generated primers. Primers should be checked for secondary structure formation and hybridisation to other primers in the scheme using a primer analyzer tool.

Once primer modifications are finalized, create new version configuration directories of the RAMPART protocol and ARTIC bioinformatics pipeline following the instructions in B.1.3.

Optimizing final concentration of individual primers.

NOTE: Perform this step only after several initial rounds of sequencing.

Refer to the “Achieving more even genome coverage” section of COVID-19 ARTIC v3 Illumina library construction and sequencing protocol V5. (https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-j8nlke665l5r/v5) for guidance before attempting.

NOTE: The protocol refers to Illumina sequencing but is fully transferable to amplicon sequencing on Nanopore platforms.

If amplicons are consistently under-represented or over-represented, increase or decrease final concentration of the primer pair respectively.

Assess the expected performance of a primer scheme against set(s) of reference sequences in silico

After generating a primer scheme, you may wish to see how well it is expected to perform against sets of sequences representing different diversity, before starting sequencing.

Use a DNA manipulation and visualization program such as ApE by Davies et. al, 2022 or nucleotide alignment software such as BLASTn suite-2Sequences to align primer oligo sequences to input sequences or use a specific multiplex primer validation tool such as the R package openPrimeR by Doring et. al, 2023.

Assess mismatches between the input sequence(s) and each primer. You may consider summing and plotting the proportion of primers in the scheme which anneal with a given number of mismatches (e.g 0, 1, 2, 3, unbound).

Primers annealing with more than 3 mismatches indicate sequence variation that could affect amplification. It may be possible to redesign individual primers to account for extra diversity (see above, section 3), or change the reference sequences to better capture the intended diversity. If representative reference sequences are not available, refer to part 1 above.

Analyse average read depth per amplicon

Analyse depth of coverage per nucleotide of the reference genome to generate coverage profiles as shown in Figure **3.4** of the main text.

NOTE: Coverage profiles inform how well the primer scheme is performing across the genome. Excessive peaks or troughs appearing consistently between several samples likely indicate a bias or lack of annealing of primer pair(s) during the amplification step. These areas should be prioritized for optimization following the guidance provided above, in section 3 of this Supplemental File.

Use the script **mappingSummary.sh** located in the directory **'artic-rabv/other-scripts/'** to generate a) summary statistics of mapped reads and b) depth of coverage per base. To use the script:

Ensure the script is made executable in your local environment using the following command: **chmod u+x /path/mappingSummary.sh**

Run the script from the directory containing the output from artic-rabv bioinformatics scripts:

cd /path/analysis/run_name

/path/artic-rabv/other-scripts/mappingSummary.sh

This script automatically finds bam files in the current directory and extract depth and coverage summary statistics that will be output in files '_mappingStats.txt' and '_depth.txt' for each sample.

Use the **depth_plots.R** script located in the directory **'artic-rabv/DEPTHS/'** to import the mappingSummary.sh outputs to R and plot.

B.1.3 Computational Set-up

This Supplementary file describes how to set up directories for running RAMPART and the ARTIC bioinformatics pipeline.

NOTE: These instructions assume the pre-existing directory structure and example templates provided in the artic-rabv repository on GitHub (https://github.com/kirstyn/artic-rabv). New version (e.g V1, V2, V3) configuration directories must be set up for both RAMPART and ARTIC for each unique version of a primer scheme (either generated by Primal Scheme or following manual modification of individual primers). Steps 2 and 3 describe configuration directory setup for RAMPART and ARTIC respectively. Step 3 describes how to set up these directories after manually editing primers.

NOTE:

Create a directory for the project in the 'artic-rabv/analysis' folder.

cd /path/artic-rabv/analysis

mkdir project_name

RAMPART protocol setup.

Instructions below detail how to customise RAMPART outputs to provide more informative visualisations for your project:

Make a copy of the example_protocol directory in 'rampart' and rename it according to your primer scheme name and version e.g. scheme_name_V1_protocol.

The table below summarizes the configuration files in this directory and how to edit them to customize RAMPART outputs for your project. An example RAMPART onscreen output is shown in Figure **3.3**.

File name	Function	How to create in scheme_name_V1_protocol

genome.json	Describes index reference genome features (e.g length, position of genes).	Open in a text editor and edit fields using information from the GenBank entry for the index reference sequence.
primers.json	Describes the location of amplicon products on the index reference genome in the format [Start,End].	Extract information from primer_scheme.insert.bed file from the Primal Scheme output. E.g create .csv from .bed file and add rest of formatting manually, copying the example in example_protocol.
protocol.json	Display and annotation options.	*Optional file*: can edit default field entries if desired.
references.fasta	Reference genome(s). The first sequence should be the index reference, after which any additional sequences may be included.	Copy reference. fasta from Primal Scheme output and rename as references.fasta. Add or remove any sequences as preferred (except the index reference sequence).
primer_file.tsv	For user reference only.	Copy from Primal Scheme output.

ARTIC bioinformatics pipeline set up.

NOTE: More information is available at (https://artic.readthedocs.io/en/latest/)

Create a new primer scheme directory and version subdirectory in ‘primer-schemes’ using the command:

mkdir -p /path/artic-rabv/primer-schemes/scheme_name/V1

Copy or create the following files into the version directory according to the table:

scheme_name.reference.fasta	Copy from Primal Scheme output.
scheme_name.primer.bed	Copy from Primal Scheme output.

NOTE: Older versions of the artic package and Primal Scheme used.scheme.bed files instead of primer.bed files. If you are using a newer version of the artic package with a primer scheme generated by an older version of Primal Scheme that only has a .scheme.bed file associated, you will need to create a symbolic link called scheme_name.primer.bed, that points to the existing scheme_name.scheme.bed file in order for it to be read by the artic package. If you are using an older version of the artic package, and a primer scheme designed using the current primer scheme website, we recommend updating your artic package, or if not possible, you can create a symbolic link called scheme_name.scheme.bed, that points to the existing scheme_name.primer.bed file.

To create a symbolic link, run the command: **ln -s existing_file link_name**.

Set up RAMPART and ARTIC directories for manually modified primer schemes.

NOTE: If individual primer sequences are manually modified in a scheme, a new version of the RAMPART and ARTIC directories must be created with manually modified configuration files to reflect the changes. Within the new version directory, modified primers must keep the same name in the format **scheme_name_X_DIRECTION** (Figure B. 1). *e.g do not add any extra details such as _v2.* If using alternative primers, these are assigned by adding '**_alt**' to the end of the primer name at the position for which the alternative is available. There is no limit to the number of alternative primers which may be assigned to a position. For more info, see (https://artic.readthedocs.io/en/latest/primer-schemes/).

Align your modified primer scheme to the same 'index reference' genome to which the original scheme is referenced (refer to Prerequisites Step 2.2), using a standard alignment tool or genome editing software.

NOTE: This step is very important as any modifications to primer positions must be reflected in the configuration file with respect to the index reference of the scheme.

Create the modified RAMPART protocol directory.

Make a copy of the original rampart protocol directory (e.g 'rampart/scheme_name_V1_protocol/' and rename it according to the new version (e.g scheme_name_V2_protocol).

Edit the primer_file.tsv with details of modified primer sequences. Add new rows for any alternative primers, adding '_alt' to the name as in the note above.

Edit the configuration file primers.json to reflect any changes to the start or end position of amplicons. New amplicon positions should be determined by referring to the primer alignment to the index reference genome generated in Step 3.1 above.

NOTE: If you have not changed the start or end position of primers, you do not need to change anything during this step.

The files genome.json and references.fasta should not be changed.

The "name" option in protocol.json may be edited to reflect the new scheme version.

Create the modified ARTIC directory.

Make a copy of the original ARTIC scheme version subdirectory (e.g 'primer-schemes/scheme_name/V1' and rename it to the next sequential version number (e.g V2).

Carefully edit the scheme_name.primer.bed configuration file to reflect any changes to the position of the modified primers. These should be determined by referring to the primer alignment to the index reference genome generated in Step 3.1 above.

NOTE: If you have not changed the start or end position of primers, you do not need to change anything during this step.

The scheme_name.reference.fasta should not be changed

B.1.4 RABV Whole Genome Sequencing Worksheet

Make sure to adjust the number of samples so that the calculations are updated below

↓

Number of samples: (including 1 NC per run) =	24

cDNA synthesis

Reagent	Volume (µl)	No of reactions plus excess	Volume required
Lunascript RT	2	26.4	52.8
Water	3		79.2

Temp	Time
25°C	2 min
55°C	10 min

Multiplex PCR amplification **Reaction volume =** **12.5**

Primer volume calculation

V1 primers	**A**	**B**
Number of primers	41	41
Calculated volume	0.8	0.8

*Calculate for final concentration of 0.015uM of each primer in the reaction

Multiplex PCR

Reagent	**Volume per reaction (Pool A) (µl)**	**Volume per reaction (Pool B) (µl)**	**No of duplicate reactions plus excess**	**Volume required (pool A)**	**Volume required (pool B)**
Q5 Hot Start High-Fidelity 2X Master Mix	6.3	6.3	26.4	165.0	165.0
Primer pool A	0.8	0.0		20.3	0.0
Primer pool B	0.0	0.8		0.0	20.3
Nuclease free water	3.0	3.0		78.7	78.7
Total	**10.0**	**10.0**			

Temp	**Time**	**Cylces**
98 °C	30 s	1
98 °C	15 s	35*
63–65 °C**	5 min	
4 °C	hold	1

*25–35 cycles. Adjust by Ct or do 33 for clinical samples and 25 for high viral load cultured samples

Touchdown (decrease from 65 °C by 0.1 °C every cycle for first 25 cycles then remaining cycles at 62.5 °C)

Normalization

Record here and add concentrations to normalization table

ST1	
ST2	

1:10 dilutions:

	NGS ID	ng/µL	size bp	nM	vol for 200 fmol*	*NFW*
	Example	***23***	*400*	*87.12*	*2.3*	*2.7*
1	**NFW**	0	*400*	*0.00*	*#DIV/0!*	*#DIV/0!*
2	S1	*4.7*	*400*	*17.80*	*11.2*	*-6.2*
3	S2	*9.42*	*400*	*35.68*	*5.6*	*-0.6*
4	S3	*6.76*	*400*	*25.61*	*7.8*	*-2.8*
5	S4	*4.82*	*400*	*18.26*	*11.0*	*-6.0*
6	S5	*4.68*	*400*	*17.73*	*11.3*	*-6.3*
7	S6	*7.64*	*400*	*28.94*	*6.9*	*-1.9*
8	S7	*8.6*	*400*	*32.58*	*6.1*	*-1.1*
9	S8	*7.28*	*400*	*27.58*	*7.3*	*-2.3*
10	S9	*8.18*	*400*	*30.98*	*6.5*	*-1.5*
11	S10	*4.94*	*400*	*18.71*	*10.7*	*-5.7*
12	S11	*7.42*	*400*	*28.11*	*7.1*	*-2.1*
13	S12	*6.54*	*400*	*24.77*	*8.1*	*-3.1*
14	S13	*10.8*	*400*	*40.91*	*4.9*	*0.1*
15	S14	*1.19*	*400*	*4.51*	*44.4*	*-39.4*
16	S15	*5.4*	*400*	*20.45*	*9.8*	*-4.8*
17	S16	*3.4*	*400*	*12.88*	*15.5*	*-10.5*

Neat:

	vol for 200 fmol	*NFW*
	0.2	*4.8*
1	*#DIV/0!*	*0.0*
2	*1.1*	*1.8*
3	*0.6*	*3.6*
4	*0.8*	*2.6*
5	*1.1*	*1.8*
6	*1.1*	*1.8*
7	*0.7*	*2.9*
8	*0.6*	*3.3*
9	*0.7*	*2.8*
10	*0.6*	*3.1*
11	*1.1*	*1.9*
12	*0.7*	*2.8*
13	*0.8*	*2.5*
14	*0.5*	*4.1*
15	*4.4*	*0.5*
16	*1.0*	*2.0*
17	*1.6*	*1.3*

18	S17	*3.04*	*400*	*11.52*	*17.4*	*-12.4*	18	*1.7*	*1.2*
19	S18	*6.92*	*400*	*26.21*	*7.6*	*-2.6*	19	*0.8*	*2.6*
20	S19	*8.54*	*400*	*32.35*	*6.2*	*-1.2*	20	*0.6*	*3.2*
21	S20	*7.04*	*400*	*26.67*	*7.5*	*-2.5*	21	*0.8*	*2.7*
22	S21	*7.00*	*400*	*26.52*	*7.5*	*-2.5*	22	*0.8*	*2.7*
23	S22	*4.62*	*400*	*17.50*	*11.4*	*-6.4*	23	*1.1*	*1.8*
24	S23	*0.796*	*400*	*3.02*	*66.3*	*-61.3*	24	*6.6*	*0.3*

*Use neat values if volume of DNA required is >5 µL/ cell color is light red

End prep of amplicons

Reagent			**Volume per reaction (µL)**	**No of reactions plus excess**	**Volume required (µL)**
NFW			3.5	26.4	92.4
Ultra II end prep reaction buffer			1		26.4
Ultra II end prep enzyme mix			0.5		13.2
Total			**5**		

Temp	**Time**
20 °C	15 min
65 °C	15 min
4 °C	1 min

Barcoding

Reagent	**Volume per reaction (µL)**	**No of reactions plus excess**	**Volume required (µL)**
Nuclease-free water	3	26.4	79.2
Blunt/TA Ligase Master Mix	5		132
Total	**8**		211.2

Temp	**Time**
20 °C	20 min
65 °C	10 min
4 °C	1 min

DNA Quantification for adapter ligation

ST1	28.77
ST2	17911.89
Library (ng/µL)	2.56
library size bp	400
Conc (nM)	9.70
vol for 200 fmol	20.63
fmol in 20 µL	193.94

<< Enter value here

nM=(ng/µL*1000000)/(660*bp)

ul=fmol/nM (eg 200/nM)

fmol=nM*µL (eg nM*20)

Adapter ligation One pot method - DO ON DAY OF SEQUENCING

OPTION 1:

NEBnext Quick ligation Module	**Vol (µL)**
Barcoded amplicon pool	20*
Nuclease free water (to make up to 20 µL)	0*
Adapter Mix II	5
NEBNext Quick Ligation Reaction Buffer (5x)	10
NEBNext Quick T4 DNA Ligase	5
Total	**20**

OPTION 2:

NEBnext Ultra II Ligation Module	**Vol (µL)**
Barcoded amplicon pool	20*
Nuclease free water (to make up to 20 µL)	0*
Adapter Mix II	5
NEBNext Ultra II Ligation Master Mix (5x)	25
NEBNext Ultra II Ligation Enhancer	0.5
Total	**50.5**

OPTION 3:

Blunt/TA ligation	**Vol (µL)**
Barcoded amplicon pool	20*
Nuclease free water (to make up to 20 µL)	0*
Adapter Mix II	5
Blunt/TA Ligase mastermix	25
Total	**50**

*If less than 20 µL is computed, add nuclease free water elution buffer to make up to 20 µL.

DNA Quantification for final library

ST1	28.77
ST2	17911.89
Library (ng/µL)	2.56
library size bp	400
Conc (nM)	9.70
vol for 50 fmol	5.16
fmol in 12 µL	116.36

<< Enter value here

nM=(ng/µL*1000000)/(660*bp)

ul=fmol/nM (eg 50/nM)

fmol=nM*µL (eg nM*12)

DNA Library mix preparation

Library Mix	**Vol (µL)**
Sequencing Buffer (SQB)	37.5
Loading Beads (LB)	25.5
DNA Library	12
Elution Buffer (to make up DNA library volume)	0
Total	75

Sequencing run

	Run details	Notes
Run number		Take this from the sequencing run log
Experiment name		Do not add spaces
Sample ID		Do not add spaces
Kit		Click on the Ligation kit used (e.g. SQK-LSK-109)
Barcoding Kit		Click on the appropriate barcoding kit used
Basecalling		can set this to on but takes a long time so is quicker to go in alpha with guppy
Barcoding		Off/on with trimming/ on without trimming
Run length (hours)	72	
Bias Voltage (mv)	-180	*Adjust if flow cell already been used (see table below)
Active Channel Selection	on	Consider changing this and the time if looking for long reads!
Time between mux scans (hours)	1.5	
Output location	/data/	This depends on how MinKNOW was installed, could just be data/
Output format	fast5, fastq	Fastq basecalling is very slow so it's probably best just to do it with guppy

Total previous runtime (h)	Voltage to set (mV) for MinION Mk 1B, Flongle, or GridION*	Voltage to set (mV) for PromethION*
12	–190	–180
24	–210	–200
36	–230	–220
48 or more	–250	–240

Initial active channel: **Total** [ ] Started sequencing with

Record the initial channels in strand vs single pore (light green vs dark green) to give an approximate pore occupancy. This number will fluctuate so just give an approximation.

Channels in Strand (light green)	
Single Pore Channels (dartk green)	
Pore Occupancy (%)	#DIV/0!

*A good library will have a higher proportion of light green than dark green and high pore occupancy %.

Monitor the run

Total Reads	amount of data you have generated
Basecall statistics	indication of the queue for the reads to be basecalled
Channels Panel	
Light Green	active ports sequencing
Dark Green	single pore channels
Dark blue	recovering, high numbers may indicate that the library needs additional clean up steps
Light blue	inactive, high numbers at the beginning of the run may indicate osmotic imbalance
	*Click on 'More' for more detail - after 30 min check that 70%+ of all active pores are in strand
The duty time	plots summarised channel states over time - use to assess the quality of the run
	Good quality library will result in most of the pores being in 'Sequencing'

	A build-up of 'Recovering' pores indicates channels being blocked and most likely the library needs more washing
	A high ratio of 'Pore' to 'Sequencing' states indicates insufficient starting material, either loss of sample or inefficient ligation of adapters
	High number of 'Inactive' channels building up over time indicates damage by air bubbles, osmotic imbalance or contaminants
Read length histogram	shows the read lengths being read; this is similar to your expected library size
Cumulative throughput	bases sequenced and basecalled and if they have passed or failed
Trace Viewer	shows the current levels from individual channels

B.1.5 Materials

Name ***(Brand name)***	**Company**	**Catalog Number**
Software		
Sequencing software *(MinKnow)*	Oxford Nanopore Technologies	https://community.nanoporetech.com/downloads
Bioinformatics tool kit *(Guppy*	Oxford Nanopore Technologies	https://community.nanoporetech.com/docs/prepare/library_prep_protocols/Guppy-protocol/v/gpb_2003_v1_revao_14dec2018
Equipment		
Thermal cycler *(miniPCR™ mini16 thermal cycler)*	Cambio	MP-QP-1016-01
Homogenizer *(Precellys Evolution Touch Homogenizer)*	Bertin Instruments	EQ02520-300
Cold Racks (0.2-0.5mL) *(PCR Mini cooler with transparent lid)*	BRAND	781260
Pipettor		
(Pipetman L Fixed F1000L, 1000 uL)	Gilson	SKU: FA10030
(Pipetman L Fixed F100L, 100 uL)	Gilson	SKU: FA10024
(Pipetman L Fixed F10L, 10 uL)	Gilson	SKU: FA10020
(Pipetman L Fixed F1L, 1 uL)	Gilson	SKU: FA10025

(Pipetman L Fixed F20L, 20 uL)	Gilson	SKU: FA10021
(Pipetman L Fixed F250L, 250 uL)	Gilson	SKU: FA10026
Fluorometer *(Qubit 4 Fluorometer)*	Thermofisher scientific/Fisher scientific	Q33238
Laptop *(Any brand with ~2 GB of drive space, minimum of 512 GB storage space, msi installer [GPU]*		
Microcentrifuge *(Refrigerated centrifuge)*	Thermofisher scientific/Fisher scientific	75004081
Vortex mixer *(Basic vortex mixer)*	Thermofisher scientific/Fisher scientific	88882011
Magnetic rack *(DynaMag -2 Magnet)*	Thermofisher scientific/Fisher scientific	12321D
Sequencing device *(MinION)*	Oxford Nanopore Technologies	MinION Mk1B
RNA Extraction		
RNA extraction kit *(Qiagen RNEasy Mini Kit 250)*	Qiagen	74106
RNA stabilizing reagents		
(RNA later)	Invitrogen	AM7020
(DNA/RNA Shield)	Zymo Research	R1100-50
PCR		
Nuclease-free Water *(Nuclease-free Water [not DEPC-treated])*	Thermofisher scientific/Fisher scientific	AM9937
Master mix for first strand cDNA synthesis *(LunaScript RT SuperMix Kit)*	New England Biolabs	E3010S

DNA amplification master mix *(Q5® Hot Start High-Fidelity 2X Master Mix [NEB])*	New England Biolabs	M0494L
Primer (Scheme) *(Custom DNA oligos)*	Invitrogen	
SPRI Bead Clean-up		
SPRI beads *(Aline Biosciences PCR Clean DX)*	Cambio	AL-AC1003-50
Ethanol, Pure Absolute, >99.8% (GC) [Riedel-De Haen]	Merck	818760
Short Fragment buffer *(SFB expansion pack)*	Oxford Nanopore Technologies	EXP-SFB001
DNA Quantification		
DNA quantification kit *(Qubit® dsDNA HS Assay Kit)*	Thermofisher scientific/Fisher scientific	Q32854
DNA quantification assay tubes *(Qubit™ Assay Tubes)*	Thermofisher scientific/Fisher scientific	Q32856
End Prep and barcoding *(Qubit™ Assay Tubes)*		
End Prep master mix *(NEBNext Ultra End Repair/dA-Tailing Module)*	New England Biolabs	E7546L
Barcoding kit		
*Chemistry 9	Oxford Nanopore Technologies	*Chemistry 9
(Native Barcoding Expansion 1-12)		EXP-NBD104
(Native Barcoding Expansion 13-24)		EXP-NBD114
(Native Barcoding Expansion 96)		EXP-NBD196
*Chemistry 14	Oxford Nanopore Technologies	*Chemistry 14

(not compatible)		(not compatible)
(Native Barcoding Kit 24 V14)		SQK-NBD114.24
(Native Barcoding Kit 96 V14)		SQK-NBD114.96
Ligation mastermix *(Blunt/TA Ligase Master Mix)*	New England Biolabs	M0367S
Adapter Ligation		
Adapter ligation master mix		
(NEBNext Quick Ligation Module)	New England Biolabs	E6056S
(NEBNext Ultra II Ligation Module)	New England Biolabs	E7595S
(Blunt/TA Ligase Master Mix)	New England Biolabs	M0367S
Adapter mix		
*Chemistry 9	Oxford Nanopore Technologies	*Chemistry 9 EXP-AMII001
(Adapter Mix II [AMII])		
*Chemistry 14	Oxford Nanopore Technologies	*Chemistry 14 EXP-NBA114
(Native adapter [NA])		
Sequencing		
Flowcell priming kit		
*Chemistry 9	Oxford Nanopore Technologies	*Chemistry 9 EXP-FLP002
(Flush Buffer [FB])		
(Flush Tether [FT])		
*Chemistry 14	Oxford Nanopore Technologies	*Chemistry 14 EXP-FLP004
(Flow Cell Flush [FCF])		
(Flow Cell Tether [FCT])		
Ligation Sequencing Kit		
*Chemistry 9	Oxford Nanopore Technologies	*Chemistry 9 SQK-LSK109
Adapter Mix *(Adapter Mix [AMX])*		

Ligation Buffer *(Ligation buffer [LNB])*		
Short Fragment Buffer *(Short Fragment buffer [SFB])*		
Sequencing Buffer *(Sequencing Buffer [SQB])*		
Elution Buffer *(Elution buffer [EB])*		
Loading Beads *(Loading Beads [LB])*		
Sequencing Tether *(Sequencing Tether [SQT])*		
*Chemistry 14	Oxford Nanopore Technologies	*Chemistry 14 SQK-LSK114
Adapter Mix *(Ligation Adapter [LA])*		
Ligation Buffer *(Ligation buffer [LNB])*		
Short Fragment Buffer *(Short Fragment buffer [SFB])*		
Sequencing Buffer *(Sequencing Buffer [SB])*		
Elution Buffer *(Elution buffer [EB])*		
Loading Beads *(Loading Beads [LIB])*		
Sequencing Tether *(Flow Cell Tether [FCT])*		
Library solution *(Library solution [LIS])*		

Flush buffer *(Flow Cell Flush [FCF])*		
Flow Cell		
*Chemistry 9 *(Flow Cell [R9.4.1])*	Oxford Nanopore Technologies	*Chemistry 9 FLO-MIN106D
*Chemistry 14 *(Flow Cell [R10.4.1])*	Oxford Nanopore Technologies	*Chemistry 14 FLO-MIN114
Flow Cell wash		
Flowcell wash kit *(Flow cell wash kit)*	Oxford Nanopore Technologies	EXP-WSH004
Consummables		
Surface decontaminant		
(DNA Away Surface Decontaminant, Squeeze Bottle [Molecular Bio])	Thermofisher scientific/Fisher scientific	7010PK
(RNase Away Surface Decontaminant, Bottle [Molecular Bio])	Thermofisher scientific/Fisher scientific	7002PK
PCR 8-Tube Strip 0.2ml, individual cap *(PCR 8-Tube Strip 0.2ml, with Individual attached Flat Caps, Sterile, DNAse/RNAse, Pyrogen Free, Natural [Greiner])*	Greiner	608281
PCR Tube 0.2ml *(PCR Tube 0.2ml, Natural [Domed Cap] Bagged in 500s, non-sterile [Greiner])*	Greiner	671201
1000µL Filter Tips (500) *(Stacked 1000µL Filter Tips [500])*	Thermofisher scientific/Fisher scientific	11977724

100µL Filter Tips (1000)	Thermofisher scientific/Fisher scientific	11947724
10µL Filter Tips (1000) *(Stacked 100µL Filter Tips [1000])*	Thermofisher scientific/Fisher scientific	11907724
Reinforced tubes (2ml) with screw caps and o-rings *(Fisherbrand™ Bulk tubes)*	Thermofisher scientific/Fisher scientific	15545809
Microcentrifuge tube (1.5ml) *(1.5 ml Eppendorf Tubes [500])*	Eppendorf	1229888
DNA LoBind Tubes (1.5ml) *(DNA LoBind Tubes)*	Thermofisher scientific/Fisher scientific	10051232
Cryobabies labels		
Gloves (S/M/L)		
Paper towel		

Appendix C:

Chapter 4 Appendix

C.1 Supplementary Files

Table C. 1. Metadata of samples included in the study.

Isolate_ID	Municipality	Province	Region	Species	MADDOG Lineage Assignment	Collection Date
Luzon Island Group						
RADDL5-005	Iriga City	Camarines Sur	Bicol Region	Canis familiaris	Asian SEA4_A1.2	30-Jan-15
153	Ligao City	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	17-May-18
REG_5_2018-50	Ligao City	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	12-Feb-18
REG_5_2018-100	Libon	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	27-Mar-18
REG_5_2018-311	Tiwi	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	13-Nov-18
REG_5_2018-304	Tiwi	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	25-Oct-18
REG_5_2019-13	Ligao City	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	05-Jul-19
REG_5_2019-1	Tabaco	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	06-Jul-19
R5-21-132	Malinao	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	15-Nov-21
R5-21-136	Libon	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	02-Dec-21

R5-21-115	Vinzons	Camarines Norte	Bicol Region	Canis familiaris	Asian SEA4_A1.2	28-Sep-21
R5-21-140	Vinzons	Camarines Norte	Bicol Region	Canis familiaris	Asian SEA4_A1.2	10-Dec-21
R5-21-128	Naga City	Camarines Sur	Bicol Region	Canis familiaris	Asian SEA4_A1.2	09-Nov-21
R5-21-119	Irosin	Sorsogon	Bicol Region	Canis familiaris	Asian SEA4_A1.2	15-Oct-21
R5-09	Malilipot	Albay	Bicol Region	Felis catus	Asian SEA4_A1.2	07-Feb-22
R5-22-008	Tabaco	Albay	Bicol Region	Canis familiaris	Asian SEA4_A1.2	02-Feb-22
R2-081	Tuguegarao City	Cagayan	Cagayan Valley	Canis familiaris	Asian SEA4_A1.1	31-Aug-20
Z-98-718	San Juan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	13-Jul-98
Z-98-1061	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1	05-Aug-98
Z-98-134	Dasmariñas	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1	09-Feb-98
Z-98-433	Kawit	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.2	30-Apr-98
Z-98-823	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.2	05-Aug-98
Z-98-206	Santa Cruz	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	02-Mar-98
Z-98-285	San Pablo City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	18-Mar-98
Z-98-335	Nagcarlan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	01-Apr-98
Z-98-667	Calamba City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	29-Jun-98

Z-98-712	Siniloan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	11-Jul-98
Z-98-780	Alaminos	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	25-Jul-98
Z-98-286	Mulanay	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1	19-Mar-98
Z-98-860	San Francisco	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1	14-Aug-98
Z-06-150	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	24-Apr-06
Z-06-295	Calatagan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_C1	22-Jul-06
Z-06-39	Tanauan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.2	26-Jan-06
Z-07-366	Lipa City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.2	12-Nov-07
Z-07-07	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	08-Jan-07
Z-07-22	Amadeo	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1	23-Jan-07
Z-07-222	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	30-Jun-07
Z-07-38	Bacoor	Cavite	Calabarzon	Canis familiaris	Asian SEA4_B1.1	02-May-07
Z-08-351	General Mariano Alvarez	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1	12-Dec-08
Z-07-398	Calamba City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	19-Dec-07
Z-08-359	San Pablo City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	25-Dec-08

Z-07-387	San Antonio	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1.2	07-Dec-07
Z-07-403	Lucena	Quezon	Calabarzon	Canis familiaris	Asian SEA4_B1.1.1	22-Dec-07
Z12-012	Tanauan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	16-Jan-12
Z-12-212	Bauan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	04-Oct-12
Z-12-012	Tanauan	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	16-Jan-12
Z-12-118	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	31-May-12
Z-12-134	Imus	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1	18-Jun-12
Z-12-060	General Mariano Alvarez	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.2	25-Feb-12
Z-12-001	Santa Rosa City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	01-Jan-12
Z-12-034	San Pablo City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	06-Feb-12
Z-13-065	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	04-Apr-13
Z-13-171	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	05-Aug-13
Z-13-184	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	29-Aug-13
Z-13-009	Tanza	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	19-Jan-13
Z-13-066	Imus	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	05-Apr-13

Z-13-089	Naic	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	04-May-13
Z-13-094	Imus	Cavite	Calabarzon	Canis familiaris	Asian SEA4_B1.1	10-May-13
Z-13-135	Indang	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	12-Jun-13
Z-13-259	Cavite City	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	18-Dec-13
Z-13-037	Cavinti	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	25-Feb-13
Z-13-081	Santa Cruz	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	25-Apr-13
Z-13-161	Calamba City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	20-Jul-13
Z-13-204	Calauan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	26-Sep-13
Z-13-234	Calamba City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	15-Nov-13
Z-13-086	Binangonan	Rizal	Calabarzon	Canis familiaris	Asian SEA4_B1.1	03-May-13
Z-14-146	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	26-Jun-14
Z-14-098	Cuenca	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	29-Apr-14
Z-14-05	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	05-Jan-14
Z-14-106	Cavite City	Cavite	Calabarzon	Felis catus	Asian SEA4_A1.1.2	08-May-14
Z-14-136	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	11-Jun-14

Z14-142	Binan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_C1.1	21-Jun-14
Z-14-149	San Pablo City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	28-Jun-14
Z-14-023	Santa Cruz	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	31-Jan-14
Z-14-142	Binan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_C1.1	21-Jun-14
Z-14-243	Santa Cruz	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1	13-Nov-14
Z-14-033	Atimonan	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1.2	07-Feb-14
Z-14-058	Taytay	Rizal	Calabarzon	Canis familiaris	Asian SEA4_B1.1	14-Mar-14
Z-15-130	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	06-Jul-15
Z-15-163	Bacoor	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	05-Sep-15
Z-15-116	San Pedro	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	17-Jun-15
Z-15-001	Pililla	Rizal	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	01-Jan-15
Z-16-159	Tanza	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	12-Sep-16
Z-16-151	Carmona	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.2	05-Sep-16
Z-16-196	Dasmariñas	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	23-Nov-16
Z-16-013	San Pedro	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	16-Jan-16

Z-16-082	Santa Rosa City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	24-May-16
Z-16-211	Binan	Laguna	Calabarzon	Canis familiaris	Asian SEA4_C1.1	19-Dec-16
Z-16-209	Candelaria	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1	14-Dec-16
Z-16-186	Antipolo City	Rizal	Calabarzon	Canis familiaris	Asian SEA4_A1.1	03-Nov-16
Z-17-046	Santo Tomas	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	06-Mar-17
Z-17-177	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	25-Sep-17
Z-17-217	Imus	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	16-Nov-17
Z-17-221	Noveleta	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	28-Nov-17
Z-17-200	Cabuyao	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	31-Oct-17
Z-17-193	Infanta	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	13-Oct-17
Z-18-114	San Jose	Batangas	Calabarzon	Canis familiaris	Asian SEA4_C1.1	28-May-18
Z-18-001	Bacoor	Cavite	Calabarzon	Felis catus	Asian SEA4_A1.1.2	02-Jan-18
Z-18-251	Santa Rosa City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	07-Dec-18
Z-19-029	Lipa City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1	26-Feb-19
Z-19-123	Imus	Cavite	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	11-Aug-19
Z-19-136	Calamba City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	05-Sep-19

BAI/BAI4A/can	Angono	Rizal	Calabarzon	Canis familiaris	Asian SEA4_A1.2	27-May-19
Z-19-88	Angono	Rizal	Calabarzon	Canis familiaris	Asian SEA4_A1.2	31-May-19
Z-20-01	Bacoor	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	03-Jan-20
Z-20-110	San Pedro	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	23-Oct-20
Z-20-51	Angono	Rizal	Calabarzon	Canis familiaris	Asian SEA4_A1.2	02-Mar-20
4A-22-104	Lipa City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_A1.2	25-Jan-22
4A-22-40	Batangas City	Batangas	Calabarzon	Canis familiaris	Asian SEA4_B1.1.1	10-Jan-22
4A-22-171	General Trias	Cavite	Calabarzon	Canis familiaris	Asian SEA4_C1.1	06-Feb-22
4A-22-203	Lucena	Quezon	Calabarzon	Canis familiaris	Asian SEA4_A1.1.2	11-Feb-22
4A-22-98	Tayabas City	Quezon	Calabarzon	Canis familiaris	Asian SEA4_B1.1.1	22-Jan-22
Z-23-01	Santa Rosa City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	03-Jan-23
Z-23-01Sa	Santa Rosa City	Laguna	Calabarzon	Canis familiaris	Asian SEA4_A1.2	03-Jan-23
Z-98-616	San Miguel	Bulacan	Central Luzon	Canis familiaris	Asian SEA4_A1.3	18-Jun-98
Z-14-182	Pulilan	Bulacan	Central Luzon	Canis familiaris	Asian SEA4_A1.2	20-Aug-14
RADDL3-1045	Limay	Bataan	Central Luzon	Canis familiaris	Asian SEA4_A1.2	23-Oct-15

RADDL3-15-172	San Jose City	Nueva Ecija	Central Luzon	Canis familiaris	Asian SEA4_A1.2	12-Feb-15
RADDL3-1038	Angeles City	Pampanga	Central Luzon	Canis familiaris	Asian SEA4_A1.2	22-Oct-15
RADDL3-845	Floridablanca	Pampanga	Central Luzon	Canis familiaris	Asian SEA4_A1.2	02-Sep-15
RADDL3-16-521	Malolos City	Bulacan	Central Luzon	Canis familiaris	Asian SEA4_A1.2	15-Jun-16
RADDL3-16-627	Malolos City	Bulacan	Central Luzon	Canis familiaris	Asian SEA4_A1.2	27-Jul-16
RADDL3-16-1008	Cabanatuan City	Nueva Ecija	Central Luzon	Canis familiaris	Asian SEA4_A1.2	27-Oct-16
RADDL3-16-468	Angeles City	Pampanga	Central Luzon	Canis familiaris	Asian SEA4_A1.2	01-Jun-16
Z-18-159	Guiginto	Bulacan	Central Luzon	Canis familiaris	Asian SEA4_A1.2	19-Jul-18
BAI/BAI3/fel	San Miguel	Bulacan	Central Luzon	Felis catus	Asian SEA4_A1.2	06-May-19
R3-731	Cabanatuan City	Nueva Ecija	Central Luzon	Canis familiaris	Asian SEA4_A1.2	09-Dec-21
RADDL1-16-154	San Carlos City	Pangasinan	Ilocos Region	Canis familiaris	Asian SEA4_A1.1.2	21-Jul-16
RADDL1-16-153	Urbiztondo	Pangasinan	Ilocos Region	Canis familiaris	Asian SEA4_A1.2	18-Jul-16
RADDL1-16-166	unknown	Pangasinan	Ilocos Region	Canis familiaris	Asian SEA4_A1.2	09-Aug-16
R1-21-148	Urbiztondo	Pangasinan	Ilocos Region	Canis familiaris	Asian SEA4_A1.1.2	10-Apr-21
Z-12-149	Pinamalayan	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	28-Jun-12

Z-13-216	Mamburao	Occidental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1	25-Oct-13
Z-17-57	San Jose	Occidental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1	29-Mar-17
4B-21-126	Calapan City	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	26-Jan-21
4B-21-29	Victoria	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	09-Feb-21
4B-21-310	Puerto Galera	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	10-Mar-21
4B-21-35	Puerto Galera	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	05-Mar-21
4B-21-412	Puerto Galera	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	12-Apr-21
4B-21-421	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	14-Apr-21
4B-21-423	Calapan City	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	23-Apr-21
4B-21-528	Puerto Galera	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	28-May-21
4B-2422	Calintaan	Occidental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	04-Feb-22
4B-21622	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	16-Feb-22
4B-22-114	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	14-Jan-22
4B-22-13	Mansalay	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	22-Apr-22
4B-22-15	Puerto Galera	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	05-Jan-22

4B-22-16	Bongabong	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	28-Apr-22
4B-22-17	Gloria	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	08-May-22
4B-22-20	Mansalay	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	19-May-22
4B-22-21	Pinamalayan	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	31-May-22
4B-22-28	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	27-Jul-22
4B-22-30	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	12-Aug-22
4B-22-31	Pola	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	18-Aug-22
4B-22-35	Roxas	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	25-Oct-22
4B-22-40	Mansalay	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	02-Dec-22
4B-2922HM	Baco	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	09-Feb-22
4B-2922JG	Bongabong	Oriental Mindoro	MIMAROPA	Canis familiaris	Asian SEA4_C1	09-Feb-22
4B-22-37	Alcantara	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	01-Oct-22
4B-22-39	Alcantara	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	21-Oct-22
4B-22-41	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	24-Nov-22
4B-22-42	Alcantara	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	26-Nov-22

4B-22-44	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	11-Dec-22
4B-22-45	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	12-Dec-22
Z-22-103	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	21-Nov-22
Z-22-119	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	28-Dec-22
Z-22-121	San Agustin	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	19-Dec-22
4B-23-01	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	20-Jan-23
4B-23-02	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	13-Jan-23
4B-23-03	San Agustin	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	22-Jan-23
4B-23-04	San Agustin	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.1.2	31-Jan-23
4B-23-05	San Agustin	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	20-Jan-23
4B-23-06	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	02-Feb-23
4B-23-07	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	07-Feb-23
4B-23-11	San Andres	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	07-Feb-23
4B-23-12	Santa Maria	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	14-Feb-23
4B-23-13	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	09-Feb-23

4B-23-15	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	16-Feb-23
4B-23-16	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	28-Feb-23
4B-23-17	Odiongan	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	01-Mar-23
4B-23-19	San Agustin	Romblon	MIMAROPA	Canis familiaris	Asian SEA4_A1.2	01-Mar-23
H-23-011Sk_12	Santa Maria	Romblon	MIMAROPA	Homo sapiens	Asian SEA4_B1	02-Feb-23
Z-98-454	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1	04-May-98
Z-98-515	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	20-May-98
Z-98-838	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	09-Aug-98
Z-04-664	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	15-Dec-04
Z-05-102	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	21-Feb-05
Z-05-191	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	10-Apr-05
Z-05-557	Makati City	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	03-Dec-05
Z-05-96	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	17-Feb-05
Z-07-351	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	25-Oct-07
Z-07-407	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	26-Dec-07

Z-09-94	Pasay City	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	08-Apr-09
Z-12-088	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	29-Mar-12
Z-12-029	Marikina	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	03-Feb-12
Z-12-208	Kalookan City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	02-Oct-12
Z-13-002	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	02-Jan-13
Z-13-020	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	02-Feb-13
Z-13-032	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	14-Feb-13
Z-13-036	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	25-Feb-13
Z-13-071	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	10-Apr-13
Z-13-073	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_B1.1	12-Apr-13
Z-13-096	Paranaque City	Metro Manila	NCR	Felis catus	Asian SEA4_B1.1	12-May-13
Z-13-098	San Juan	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1	13-May-13
Z-13-134	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	10-Jun-13
Z-14-138	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	16-Jun-14
Z14-152	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	02-Jul-14

Z-14-101	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	04-May-14
Z-14-126	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	31-May-14
Z-15-148	Taguig	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1	04-Aug-15
Z-15-119	Pasay City	Metro Manila	NCR	Canis familiaris	Asian SEA4_C1.1	19-Jun-15
Z15-185	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	18-Oct-15
Z-15-019	Parañaque	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	28-Jan-15
Z-15-009	Quezon City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	16-Jan-15
Z-16-150	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	05-Sep-16
Z-16-210	Las Piñas	Metro Manila	NCR	Canis familiaris	Asian SEA4_C1.1	16-Dec-16
Z-17-174	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	23-Sep-17
Z-18-224	Kalookan City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	28-Oct-18
BAI/BAINCR/can	Kalookan City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	26-Jun-19
BAI/BAINCR/can	Quezon City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	28-May-19
BAI/BAINCR/can	Pasay City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	31-May-19
Z-20-24	Muntinlupa	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.2	14-Feb-20

Z-20-33	Pasay City	Metro Manila	NCR	Canis familiaris	Asian SEA4_A1.1.2	18-Feb-20
Mindanao Island Group						
18-179	Cotabato	Cotabato	Bangsamoro	Canis familiaris	Asian SEA4_A1.2	01-Sep-18
R11-21-61	Midsayap	Cotabato	Bangsamoro	Canis familiaris	Asian SEA4_A1.2	04-Oct-21
18-107	Compostela Valley	Compostela Valley	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-142	Nabunturan	Compostela Valley	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-162	Samal	Davao del Norte	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18
19-050	Panabo City	Davao del Norte	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Apr-19
R11-21-63	Tagum	Davao Del Norte	Davao Region	Canis familiaris	Asian SEA4_A1.2	08-Nov-21
18-092	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-094	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-096	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-099	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-101	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-102	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-103	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18

18-106	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-114	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-116	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-118	Digos City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jun-18
18-120	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-121	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-130	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-133	Tagum	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-135	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-138	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-144	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Jul-18
18-147	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18
18-151	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18
18-153	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18
18-163	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18

18-164	Digos City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Aug-18
18-172	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Sep-18
18-174	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Sep-18
18-181	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Sep-18
18-183	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Sep-18
18-188	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Oct-18
18-191	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Oct-18
18-194	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Oct-18
18-196	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Oct-18
18-199	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Oct-18
18-209	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Nov-18
18-214	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Nov-18
18-216	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Nov-18
18-221	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Nov-18
18-223	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Dec-18

18-224	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Dec-18
18-225	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Dec-18
19-004	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	15-Jan-19
19-011	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	16-Jan-19
19-015	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	15-Jan-19
19-022	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Feb-18
19-045	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	01-Apr-19
R11-22-002	Davao City	Davao del Sur	Davao Region	Canis familiaris	Asian SEA4_A1.2	05-Jan-22
R10-688	Malaybalay City	Bukidnon	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	02-Jul-21
R10-907	Libona	Bukidnon	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	25-Oct-21
R10-711	Cagayan de Oro City	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	12-Jul-21
R10-714	Opol	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	11-Jul-21
R10-824	Cagayan de Oro City	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	02-Sep-21
R10-856	Manticao	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	27-Sep-21
R10-920	Cagayan de Oro City	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	26-Oct-21

R10-989	Gingoog City	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	25-Nov-21
R10-990	Gingoog City	Misamis Oriental	Northern Mindanao	Canis familiaris	Asian SEA4_A1.2	25-Nov-21
R9-105	Labason	Zamboanga Del Norte	Zamboanga Peninsula	Canis familiaris	Asian SEA4_A1.2	09-Dec-21
R9-010	Zamboanga City	Zamboanga Del Sur	Zamboanga Peninsula	Felis catus	Asian SEA4_A1.2	27-Jan-20
R9-074	Vincenzo A. Sagun	Zamboanga Del Sur	Zamboanga Peninsula	Canis familiaris	Asian SEA4_A1.2	20-Aug-21
R9-089	Ipil	Zamboanga Sibugay	Zamboanga Peninsula	Canis familiaris	Asian SEA4_A1.2	07-Oct-21
Visayas Island Group						
R7-21-161	Lapu-Lapu City	Cebu	Central Visayas	Canis familiaris	Asian SEA4_A1.2	18-Oct-21
R6-21-4511	Tibiao	Antique	Western Visayas	Canis familiaris	Asian SEA4_A1.2	08-Oct-21
R6-4568	Buenavista	Guimaras	Western Visayas	Canis familiaris	Asian SEA4_A1.2	13-Oct-21
R6-21-4375	Sara	Iloilo	Western Visayas	Canis familiaris	Asian SEA4_A1.2	01-Oct-21
R6-21-4600	Pavia	Iloilo	Western Visayas	Canis familiaris	Asian SEA4_A1.2	14-Oct-21
R6-4342	Oton	Iloilo	Western Visayas	Bos taurus	Asian SEA4_A1.2	30-Sep-21
Unknown source						
KX148259	unknown	unknown	unknown	Canis familiaris	Asian SEA4_A1	23-Jun-94
KX148260	unknown	unknown	unknown	Canis familiaris	Asian SEA4_A1	27-Jul-94

KX148261	unknown	unknown	unknown	Canis familiaris	Asian SEA4_A1	29-Jun-94
KX148262	unknown	unknown	unknown	Canis familiaris	Asian SEA4_A1	05-Jul-94
KX148263	unknown	unknown	unknown	Canis familiaris	Asian SEA4_A1	28-Jun-94

Appendix D:

Chapter 5 Appendix

D.1 Supplementary Files

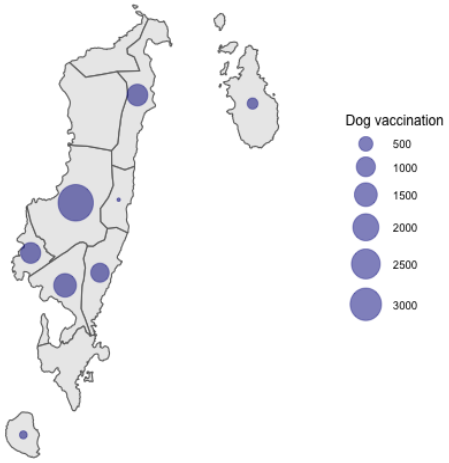


Figure D. 1. Number of dogs vaccinated per municipality in Romblon Province (Sept 2022- Sept 2023)

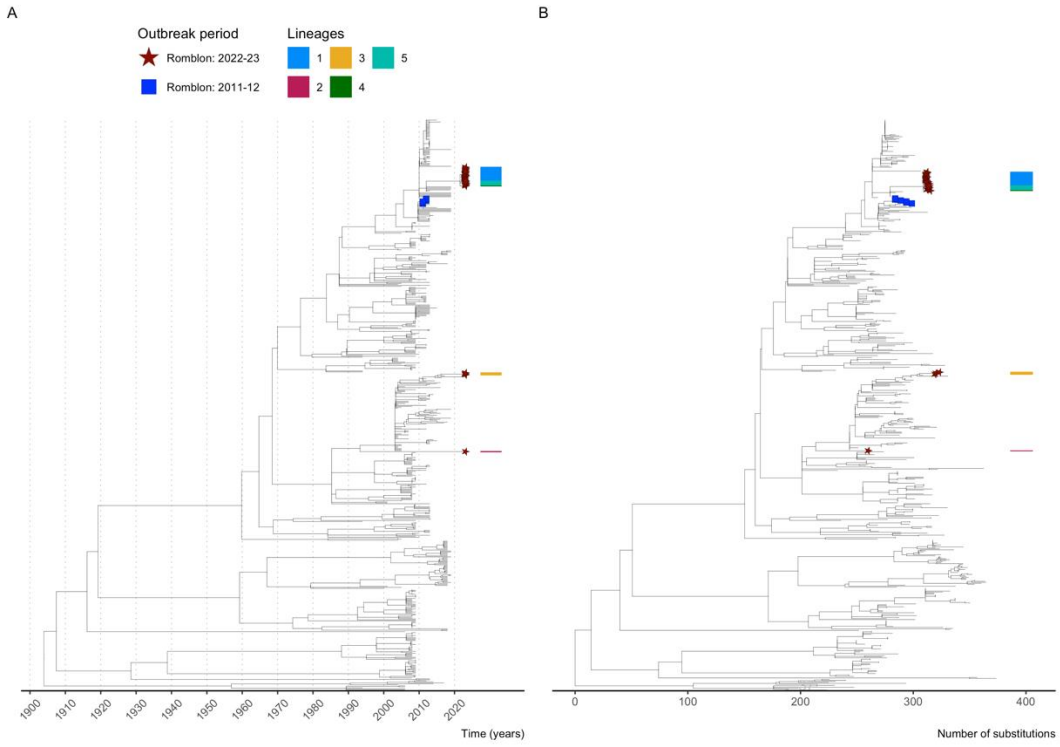


Figure D. 2. Time-scaled and substitution-scaled phylogenies from publicly available Philippines RABV sequences. A) Time-scaled and B) substitution-scaled maximum likelihood trees of 518 sequences (211-11797bp) from the Philippines spanning 1998 to 2023. The phylogenetic placement of Romblon cases from historical (2011-12) and current (2022-23) outbreaks are highlighted, as are the genetic lineages described in the main text. The top cluster from the 2022-23 Romblon outbreak represents the cluster A1 as shown in **Figure** 5.4, while the middle cluster is C1, and the lower cluster is the human case B1.

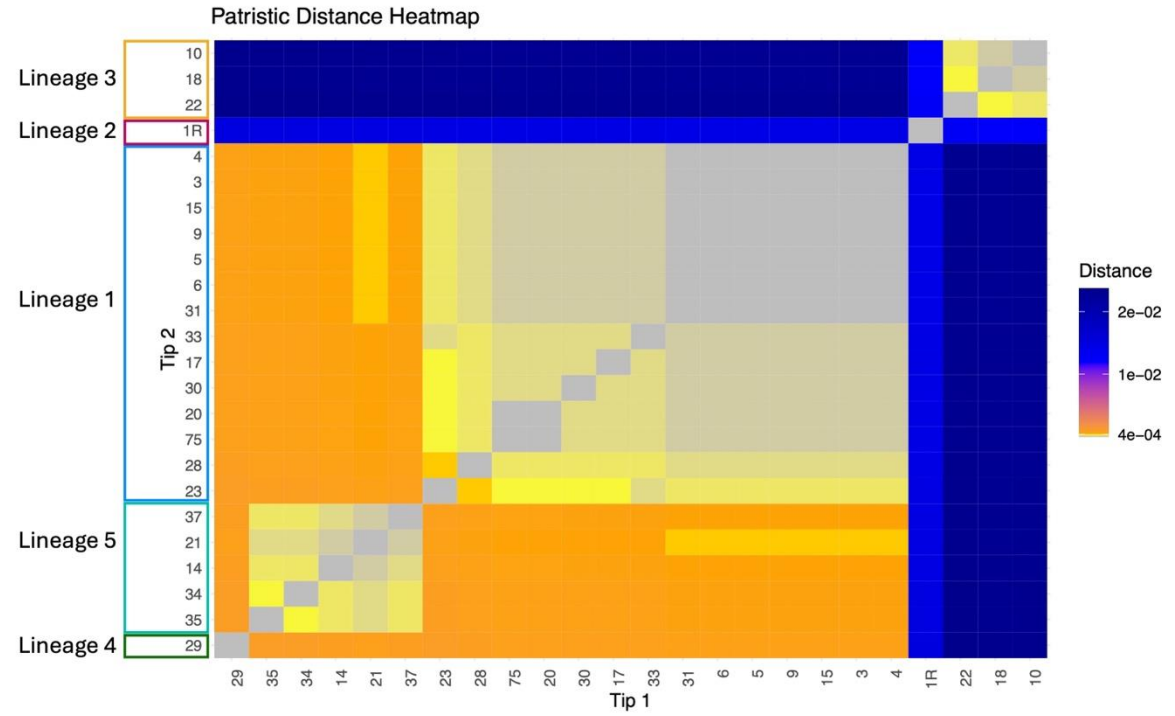


Figure D. 3. Patristic distance heatmap of Romblon sequences. The heatmap illustrates the genetic distances between sequences from Romblon, with distances calculated using the patristic method. The colour gradient runs from grey to yellow to dark blue with shades of blue representing higher genetic distances, indicating less similarity, while yellow/orange shades indicate smaller distances. The transition from yellow to orange marks the 0.0004 threshold used to delineate lineages, which are annotated on the y axis.

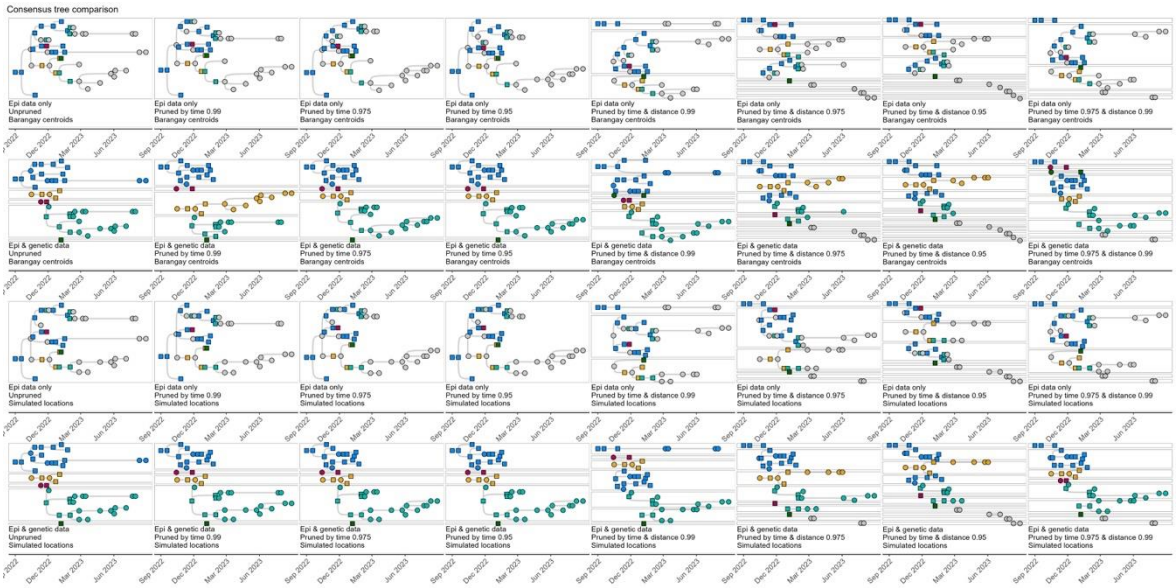
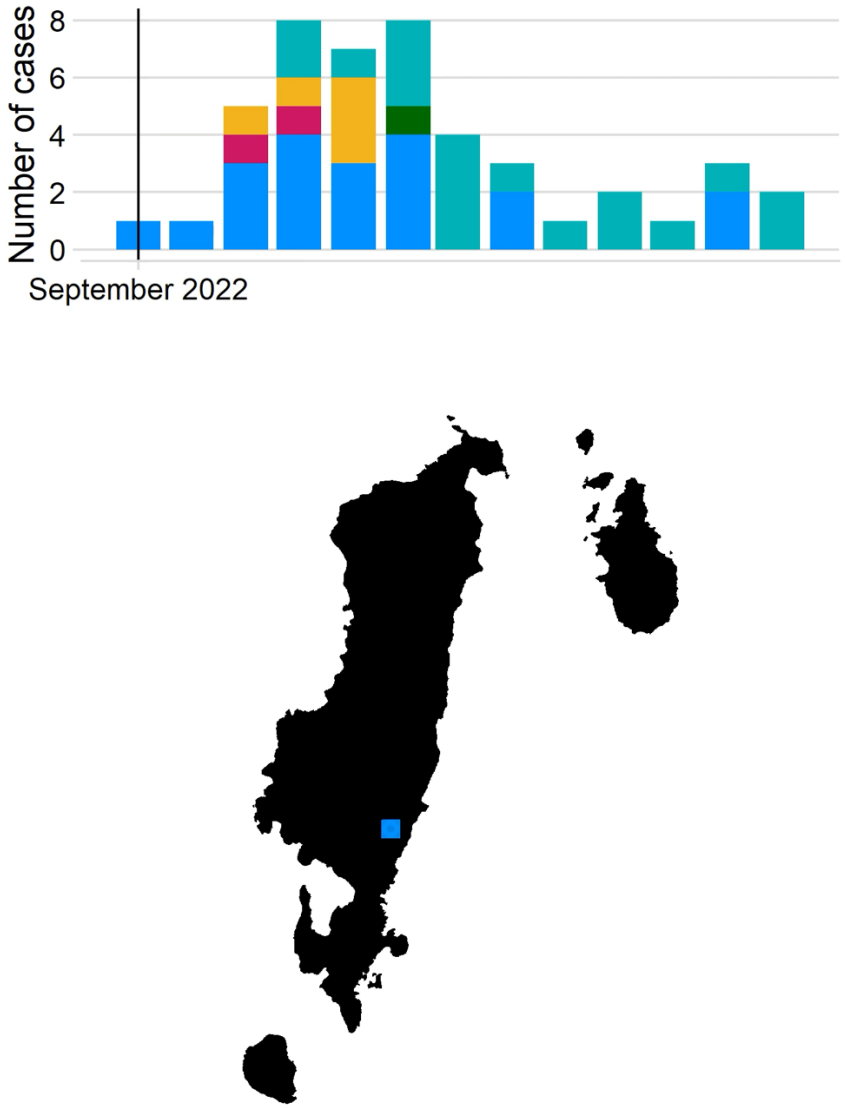


Figure D. 4. Consensus transmission tree reconstructions under different pruning thresholds and assumptions about case locations. Row 1) trees constructed using epidemiological data only and *barangay* centroids to represent case locations; Row 2) trees constructed from epidemiological data consistent with phylogenetic assignments (according to the three introductions and subsequent divergence of cluster 1 into three genetic lineages, with *barangay* centroid locations); Rows 3) and 4) trees constructed as per Rows 1 and 2 but using case locations simulated in proportion to human population density. Columns 1-8 represent pruning thresholds, specifically 1) unpruned, 2-5) pruned by the 99th, 97.5th and 95th percentile of the serial interval; and 5-7) by the 99th, 97.5th and 95th percentiles of the serial interval and dispersal kernel, and 8) by the 97.5th

and 99th percentiles of the serial interval and the dispersal kernel respectively. Squares represent sequences coloured according to lineage, and circles represent detected cases without sequences, coloured by lineage when assigned.



Movie D. 1. Animation of outbreak transmission chains. Monthly confirmed and probable dog cases coloured by genetic lineage (as per Fig. 4) shown together with the map of inferred transmission links. Squares represent sequenced cases, and circles unsequenced (unsampled) cases, except for case 45 which was not sequenced but assigned to lineage 2 based on its epidemiological link to the sequenced human case (not shown). The illustrated chains are from the consensus transmission tree with case locations simulated in proportion to human population density and pruning by the 99th percentiles of the serial interval and dispersal kernel.

Table D. 1. Whole genome sequences used in the phylogeography

Case ID	Lineage	Cluster	Municipality	Species	Collection Date
9	1	1	Alcantara	*Canis familiaris*	01/10/2022
4	1	1	Alcantara	*Canis familiaris*	21/10/2022
3	1	1	Santa Maria	*Canis familiaris*	21/11/2022
5	1	1	Odiongan	*Canis familiaris*	24/11/2022
6	1	1	Alcantara	*Canis familiaris*	26/11/2022
15	1	1	Odiongan	*Canis familiaris*	11/12/2022
75	1	1	Santa Maria	*Canis familiaris*	12/12/2022
14	5	1	Odiongan	*Canis familiaris*	28/12/2022
20	1	1	Santa Maria	*Canis familiaris*	20/01/2023
17	1	1	Odiongan	*Canis familiaris*	13/01/2023
21	5	1	San Agustin	*Canis familiaris*	20/01/2023
23	1	1	Santa Maria	*Canis familiaris*	02/02/2023
29	4	1	San Andres	*Canis familiaris*	07/02/2023
28	1	1	Santa Maria	*Canis familiaris*	07/02/2023
33	1	1	Santa Maria	*Canis familiaris*	14/02/2023
31	1	1	Odiongan	*Canis familiaris*	09/02/2023
30	1	1	Odiongan	*Canis familiaris*	16/02/2023
34	5	1	Odiongan	*Canis familiaris*	28/02/2023
35	5	1	Odiongan	*Canis familiaris*	01/03/2023
37	5	1	San Agustin	*Canis familiaris*	01/03/2023
1-R	2	2	Santa Maria	*Homo sapiens*	02/02/2023
10	3	3	San Augustin	*Canis familiaris*	19/12/2022
18	3	3	San Agustin	*Canis familiaris*	22/01/2023
22	3	3	San Agustin	*Canis familiaris*	31/01/2023

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