MOLECULAR EPIDEMIOLOGY OF AFRICAN SWINE FEVER VIRUS IN SELECTED COUNTRIES OF EASTERN AND SOUTHERN AFRICA

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

In recent decades, the growing domestic pig population in response to an increase in demand for meat in sub-Saharan Africa has been followed by the increase of African swine fever (ASF) outbreaks. African swine fever is a contagious viral disease that causes high mortality, approaching 100%, in domestic pigs and wild boars. The disease has neither cure nor a vaccine and it is caused by ASF virus (ASFV), the only member of the family *Asfarviridae*, genus *Asfivirus*, and the only known DNA arbovirus. Twenty four genotypes of ASFV have been described to date and all of them are present in Africa. Thirty three (33) African countries, South of the Sahara are known to have experienced ASF outbreaks and it continues to impact negatively on small-scale domestic pig farmers particularly in eastern and southern Africa where three ASFV transmission cycles are found and this area is believed to play an important role in the maintenance and spread of ASF. African swine fever, by its recent spread beyond its traditional geographic boundaries to Caucasus, European Union, Russia and Asia particularly to China, which is the major pork producing country, is threatening global food and nutritional security. African swine fever outbreaks are mandated to be reported to the World Organization for Animal Health (OIE) and despite the perceived increase of the reported ASF outbreaks in domestic pigs, our understanding of the process of ASFV maintenance and spread in eastern and southern Africa is quite limited. This study was conducted to investigate molecular epidemiology of the ASFV in selected countries of eastern and southern Africa. The overriding research question for this study was "How are the ASFV strains circulating and causing ASF outbreaks in eastern and southern Africa related in terms of genotypes as determined by molecular characterization, in time and space?" Phylogeographic approach applied to ASFV p72 (*B646L*) gene sequences dataset was used to assess the evolutionary history and the dispersal pattern of the ASFV strains circulating between Tanzania and its eight neighbouring countries. Furthermore, partial genetic characterization of the ASFV strains responsible for recent ASF outbreaks in Burundi and Malawi was carried out targeting specific genomic regions using standardized molecular approach. The whole genome sequencing of the ASFV strains responsible for the 2018 outbreak in Burundi and 2019 ASF outbreak in Malawi were performed using Illumina next-generation sequencing (NGS) platform. The sequencing data were subjected to bioinformatics analysis using appropriate softwares and tools. The results of this study indicate that from 2005 to 2019, a total of 1588 ASF outbreaks affecting 341 742 cases that led to 302 739 domestic pig deaths were reported to the World Organization for Animal Health (OIE) by Tanzania and

its eight neighbouring countries. The case fatality rates (CFR) varied from 15.41 to 98.95% with an overall CFR of 88.58%. Fifteen different p72 ASFV genotypes were reported by Tanzania and its eight neighbouring countries and the time to the most recent common ancestor (TMRCA) for ASFV strains dated back to 1652.233 (1626.473, 1667.735) with an evolutionary rate of 4.805×10^{-5} (2.5857×10^{-5}, 9.7789×10^{-5}). Phylogeographic dispersal analysis revealed several transboundary spread events of ASFV strains between these countries. Phylogenetic analysis of the Burundian 2018 ASFV grouped the virus within p72 (*B646L*) genotype X and clustered together with those previously reported during the 1984 and 1990 ASF outbreaks in Burundi with high nucleotide identity to some ASFV strains previously reported in neighbouring east African countries, indicating a regional distribution of this ASFV genotype. The results of this study confirmed an ASF outbreak in Karonga district in northern Malawi in September 2019. The virus was closely related to other p72 (*B646L*) genotype II ASFV strains that caused outbreaks in neighbouring eastern and southern African countries, emphasizing the possible regional transboundary transmission of this ASFV genotype. Complete genome sequencing of ASFV that caused outbreak in 2018 in Burundi (BUR/18/Rutana) and the ASF outbreak during 2019 in Malawi (MAL/19/Karonga) was conducted during this study. The complete nucleotides sequence of the ASFV BUR/18/Rutana and MAL/19/Karonga were 176 564 and 183 325 base pairs long with GC content of 38.62 and 38.48%, respectively. The MAL/19/Karonga virus had a total of 186 open reading frames (ORFs) while the BUR/18/Rutana strain had 151 ORFs. After comparative genomic analysis, the MAL/19/Karonga virus showed greater than 99% nucleotide identity with other complete nucleotides sequences of p72 genotype II viruses previously described in Europe and Asia including the Georgia 2007/1 isolate. The Burundian ASFV BUR/18/Rutana exhibited 98.95 to 99.34% nucleotide identity with p72 genotype X ASFV previously described in Kenya and in Democratic Republic of the Congo (DRC). Analysis of the *EP402R* gene that encodes the CD2v major ASFV antigen protein revealed high nucleotide sequence variation and the serotyping results classified the BUR/18/Rutana and MAL/19/Karonga ASFV strains in serogroups 7 and 8, respectively. The results of this study suggest persistent circulation of ASFV in countries involved in this study and provide important insights into the genetic structure and antigenic diversity of ASFV strains circulating in Burundi and Malawi. This is important in order to understand the transmission dynamics and genetic evolution of ASFV in eastern and southern Africa, with an ultimate goal of designing an efficient risk management strategy against ASF transboundary spread. The findings of this study advocate for more research

on ASFV sylvatic cycle and more whole genome sequencing of ASFV to improve our understanding of the transmission dynamics of the virus, structural and antigenic diversity of ASFV. These findings call for a concerted regional and international effort to control the spread of ASF in order to improve nutritional and food security.

DECLARATION

I, **JEAN NEPOMUSCENE HAKIZIMANA**, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for a degree award in any other institution.

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01 November 2021
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DEDICATION

This work is dedicated to my wife Francoise Mujawimana and my daughters Julia Ineza and Jiana Irakoze in recognition of their love, understanding, prayers and support in their own special way during the entire period of my study.

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LIST OF ABBREVIATIONS AND SYMBOLS

%:	Percentage sign
°C:	Degree Celsius
AbALV:	Abalone asfa-like virus
AIC:	Akaike Information Criterion
ASF:	African swine fever
ASFV:	African swine fever virus
ASFLI:	ASFV-like integrated elements
BLASTn:	Basic Local Alignment Search Tool for Nucleotides
BLASTp:	Basic Local Alignment Search Tool for Proteins
bp:	Base pair
BWA:	Burrows-Wheeler Aligner
CFR:	Case fatality rate
CVR:	Central variable region
DNA:	Deoxyribonucleic acid
DRC:	Democratic Republic of the Congo
GATU:	Genome Annotation Transfer Utility
GB:	Gigabyte
GTR:	General Time Reversible model
ICIPE:	International Centre of Insect Physiology and Ecology
IFS:	International Foundation for Sciences
IGR:	Intergenic region
iTOL:	Interactive Tree Of Life
Kbp:	Kilobase pairs
LSD:	Least Square Dating
LVR:	Left variable region
MAFFT:	Multiple Alignment using Fast Fourier Transform
MEGA:	Molecular Evolutionary Genetics Analysis;
MEM:	Maximum Exact Match
MGF:	Multigene families
MPPA:	Marginal Posterior Probabilities Approximation
NCBI:	National Center for Biotechnology Information
NGS:	Next-generation sequencing
OIE:	World Organization for Animal Health

ORFs:	Open reading frames
p72:	African swine fever major capsid protein
PASET:	Partnership for Skills in Applied Sciences, Engineering and Technology
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PRISMA:	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QCRI:	Qatar Computing Research Institute
QGIS:	Quantum Geographic Information System
qPCR:	Real-time Polymerase Chain Reaction
QUAST:	Quality Assessment Tool for Genome Assemblies
RNA:	Ribonucleic Acid
RSIF:	Regional Scholarship and Innovation Fund
RVR:	Right variable region
SACIDS:	Southern Africa Centre for Infectious Diseases Surveillance
SMS:	Smart Model Selection
SUA:	Sokoine University of Agriculture
TMRCA:	Time to the most recent common ancestor
TRS:	Tandem repeat sequences
USA:	United States of America
V:	Volt
WAHIS:	World Animal Health Information System
WGS:	Whole Genome Sequencing
µL:	Microliter

CHAPTER ONE

General Introduction

1.1 Background

The African swine fever virus (ASFV) belongs to the genus *Asfivirus*, family *Asfarviridae* and causes the African swine fever (ASF) a contagious viral transboundary animal disease of swine which causes high mortality, approaching 100% in naïve population of domestic pigs and Eurasian wild boars (Alonso *et al*., 2018; Dixon *et al*., 2019). African swine fever is endemic in many countries of Africa, south of the Sahara and in Sardinia (Italy) and in recent years, it has become more prevalent in European and Asian countries (Galindo and Alonso, 2017; Ge *et al*., 2018; Li *et al*., 2019). The disease is considered as the most serious constraint to domestic pig production, food and nutritional security, and livelihood of both commercial and smallholder domestic pig farmers due to its high mortality rate and associated trade restriction of domestic pigs and pork products across countries (Costard *et al*., 2009).

African swine fever virus is the only member of the family *Asfarviridae,* genus *Asfivirus*. Recently, a potential new member of the *Asfarviridae* family has been described and designated as Abalone asfa-like virus (AbALV) (Matsuyama *et al*., 2020). Until 1984, the ASFV was classified in the family of I*ridoviridae,* afterward, it was removed and placed in an unassigned genus of African swine fever virus group and in 1998, the genus name was changed to *Asfivirus* within the novel family of *Asfarviridae*, the family name coming from African swine fever virus and related viruses (Alonso *et al*., 2018). Based on its morphology, the ASFV shares some similarities with *Iridoviridae* and with *Poxviridae* if the structure of the genome and the strategy of replication are considered (Couacy-Hymann, 2019). Similarly to *Poxviridae,* the ASFV replication occurs mainly in the cytoplasm of infected cells but also an early stage of replication has been reported to occur in the nucleus (Sánchez-Vizcaíno *et al*., 2019). The length of the ASFV genomes varies between 170 and 194 kilobase pairs (kbp) with a conserved central region of about 125 kbp, in addition to the left variable region (LVR) of 38 to 47 kbp and the right variable region (RVR) of 13 to 16 kbp (de Villiers *et al*., 2010). The variation of the genome lengths of different ASFV strains is caused by the gain or loss of members of the five different multigene families (MGF) of the ASFV found in the LVR and the RVR, for instance, MGF 100, MGF 110, MGF 300, MGF 360 and MGF 530/505 (Alonso *et al*.,

2018). The virions of ASFV have an icosahedral symmetry with a linear double stranded DNA.

Based on partial nucleotides amplification and sequencing of the p72 (*B646*) gene, 24 genotypes of ASFV have been described and all these 24 genotypes are present in Africa (Lubisi *et al*., 2005; Achenbach *et al*., 2017; Quembo *et al*., 2018). The epidemiology of ASF is complex, transmission is direct and vector-borne, and the disease has well recognized sylvatic, domestic and wild boar-habitat cycles (Chenais *et al*., 2018; Penrith *et al*., 2019).

The complex epidemiology of ASF in eastern and southern Africa where all 24 described ASFV genotypes exist, necessitates the use of molecular epidemiology to support control and prevention strategies. Accurate ASFV molecular data provide information on viral genetic variation required for the development of effective control and prevention strategies, including vaccine, diagnostic test and antiviral treatment development (Arabyan *et al*., 2019; Torresi *et al*., 2020; Bao *et al*., 2021). Besides, analysis of specific genomic regions of ASFV have been proven useful to track the spread of the virus during outbreaks (Simulundu *et al*., 2017). While proof of existence of a persistent regional domestic pig cycle of ASFV is still outstanding (Mwiine *et al*., 2019; Yona *et al*., 2020), laboratory diagnosis and genotypic characterization of ASFV have been limited to some countries. The most recent genotyping studies of ASFV in eastern and southern Africa are from Democratic Republic of the Congo (DRC) (Mulumba–Mfumu *et al*., 2017; Bisimwa *et al*., 2020), Tanzania (Misinzo *et al*., 2014; Yona *et al*., 2020), Mozambique (Quembo *et al*., 2018), Zambia (Simulundu *et al*., 2017) and Uganda (Mwiine *et al*., 2019). Despite; the transboundary character of ASF, each of these studies has been limited to a single country, and our understanding of the epidemiology of ASF at the regional level is quite limited. The ASF situation in Burundi and Malawi is poorly documented with 103 015 ASF cases that led to 90 833 domestic pig deaths in both countries reported to the World Organization for Animal Health (OIE) between 2005 and 2019 (OIE, 2020). As the ASF reporting system in most African countries is characterized as incomplete and sporadic (Mulumba-Mfumu *et al*., 2019; Penrith *et al*., 2019), the real number of ASF outbreaks that occurred in Burundi and Malawi during the same period may be higher than those reported to OIE.

1.2 Problem Statement and Justification of the study

Despite the regular ASF outbreaks in domestic pigs in Burundi and Malawi, molecular characterization of the causative viruses is limited. For instance, most of the ASFV strains genetically characterized in Burundi and Malawi were collected more than two decades ago reporting ASFV p72 genotypes V, VIII and XII in Malawi and genotype X in Burundi (Lubisi *et al*., 2005; Nix *et al*., 2006). Furthermore, the complete genome sequences of ASFV strains circulating in eastern and southern Africa are quite limited. For instance, although complete genome sequences of ASFV belonging to genotypes V and VIII in Malawi have been reported, there is no ASFV p72 genotype II strain from Malawi that has been subjected to complete genome sequencing and no ASFV strain from Burundi that has been fully sequenced. To improve our understanding of the transmission and dissemination pathways of ASFV in eastern and southern Africa, genetic characterization by partial and complete genome sequencing of ASFV strains causing outbreaks in the region is required. If the genetic structure and antigenic diversity of ASFV strains causing outbreaks in the region are well understood, this information will be important in order to understand the transmission dynamics, genetic evolution of ASFV in eastern Africa, to design reliable diagnostic tools and to develop efficient and sustainable disease control strategies against ASF transboundary spread.

1.3 Research Questions

i. What are the relationship and dispersal patterns of ASFV genotypes circulating between Tanzania and its eight neighbouring countries?
ii. What are the genetic profile and transmission pattern of ASFV genotypes circulating in Malawi?
iii. What are the genotypes and phylogenetic relationship of ASFV genotypes causing recent outbreaks in Burundi?
iv. What are the complete genetic structure and antigenic diversity of ASFV genotypes circulating in Burundi and Malawi?

1.4 Study Objectives

1.4.1 General objective

The general objective of this study was to determine the genetic variation and epidemiology of ASFV in selected countries of eastern and southern Africa, for better understanding the transmission dynamics, distribution and possible control strategies.

1.4.2 Specific objectives

i. To assess the evolutionary history and the dispersal pattern of the ASFV genotypes circulating between Tanzania and its eight neighbouring countries from 2005 to 2019.

ii. To examine the genetic profile and transmission pattern of ASFV responsible for the 2019 outbreak in Malawi using a standardized molecular approach.

iii. To determine the phylogenetic characteristics of the ASFV from the 2018 outbreak in south-eastern Burundi based on partial genome amplification and nucleotide sequencing of specific viral genomic regions.

iv. To determine the genetic and antigenic diversity of ASFV responsible for outbreaks in 2018 in Burundi and 2019 in Malawi by analyzing the complete genome sequences using the Illumina next-generation sequencing platform.

CHAPTER TWO

Paper One

African Swine Fever Virus Circulation between Tanzania and Neighboring Countries: A Systematic Review and Meta-Analysis

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viruses

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Review

African Swine Fever Virus Circulation between Tanzania and Neighboring Countries: A Systematic Review and Meta-Analysis

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Abstract: For over 100 years after the description of the first case of African swine fever (ASF) in Kenya, ASF virus (ASFV) cross-border spread in eastern and southern Africa has not been fully investigated. In this manuscript, we reviewed systematically the available literature on molecular epidemiology of ASF in Tanzania and its eight neighboring countries in order to establish the transmission dynamics of ASFV between these countries. Data were retrieved from World Animal Health Information System (WAHIS), Google Scholar, PubMed, Scopus, and CrossRef databases, using the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and reviewed to document ASF outbreaks and ASFV genotypes distribution. Using phylogeographic approach applied to ASFV p72 sequence dataset, the evolutionary history and the dispersal pattern of the ASFV strains were assessed. From 2005 to 2019, a total of 1588 ASF outbreaks affecting 341,742 cases that led to 302,739 domestic pig deaths were reported. The case fatality rates (CFR) varied from 15.41% to 98.95% with an overall CFR of 88.58%. Fifteen different p72 ASFV genotypes were reported and the time to the most recent common ancestor (TMRCA) for ASFV strains dated back to 1652.233 (1626.473, 1667.735) with an evolutionary rate of 4.805×10^{-5} (2.5857×10^{-5}, 9.7789×10^{-5}). Phylogeographic dispersal analysis revealed several transboundary spread events of ASFV strains between these countries. These results suggest persistent circulation of ASFV in these countries and advocate for more research to improve our understanding of the transmission dynamics of the virus and for a regional approach to mitigate the spread of ASFV.

Keywords: African swine fever virus; phylogeography; ancestral character reconstruction; Eastern Africa

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1. Introduction

African swine fever (ASF) threatens global domestic pig industry and wild boars due to its high mortality rate, trade restriction [1] and hinders poverty alleviation, an important component of Sustainable Development Goals of the United Nations in affected countries. African swine fever was first described in Kenya in 1921 [2] and since then it has become endemic in many countries of Africa, South of the Sahara where it threatens food security and livelihoods of poor and marginalized communities keeping domestic pigs for subsistence [3–5]. The first wave of ASF outside Africa started in Portugal in 1957 and in 1960 with subsequent spread to Iberian Peninsula, other countries of the European Union, the Caribbean and Brazil before its eradication in these countries in 1990 with the

exception of Sardinia, Italy where it remains endemic since 1978 [6–8]. The second wave of ASF in Europe started with an introduction of the virus in Georgia in 2007 and from there, ASF expanded its geographical distribution to Caucasus region, European Union countries, and later on in August 2018 to Asian countries including China [1,9–11]. The expanding geographical distribution of ASF poses a threat to ASF-free countries worldwide and the maintenance of ASF in countries where domestic pigs are kept for subsistence including eastern and southern African countries is believed to fuel the global spread and risk of ASF [1,12]. For instance, the first spread of ASF virus (ASFV) genotype I to Portugal in 1957 was suspected to come from Angola while Madagascar is speculated to be the origin of the ASFV genotype II introduction into Georgia in 2007 [8,13].

African swine fever is caused by the ASF virus (ASFV), a linear double-stranded DNA arbovirus with an icosahedral morphology that was once included in the family of *Iridoviridae*, but is now assigned to the genus *Asfivirus*, family *Asfarviridae* [14], being the only member of this family and the only known DNA arbovirus. Depending on the viral isolate, the ASFV genomes vary in length from about 170 to 193 kilobase pairs and encode between 151 and 167 open reading frames with a conserved central region of about 125 kbp and variable termini [15]. These variable ends encode five multigene families (MGF) that contribute to the variability of the virus genome. Molecular characterization of distinct genome regions of ASFV has proved to be very useful in elucidating the origin and transmission pathways of ASF during outbreaks [16,17]. Different genomic regions have been targeted to detect ASFV phylogenetic relationships with different levels of precision [16]. The current approach for investigating the molecular epidemiology of ASF is through sequencing of the C-terminal end of p72 (*B646L*) gene encoding the p72 major capsid protein in order to determine the viral genotype [18]. So far, 24 ASFV p72 genotypes (I–XXIV) have been identified [19,20]. Further discrimination into subgroups of closely related viruses is usually conducted by sequence analysis of the tandem repeat sequences (TRS) located in the central variable region (CVR) within the *B602L* gene [21–23] and the intergenic region between the *I73R* and *I329L* genes [24,25]. Several other genomic regions such as the *E183L* encoding the p54 protein, the *CP204L* encoding the p30 protein, and the *EP402R* gene encoding the CD2v protein, have proved to be useful tools for molecular epidemiological and virus spread investigations [13,21,26].

Four transmission cycles have been described for ASFV: (1) a sylvatic cycle where the virus asymptomatically circulates between wild suids (mainly warthogs, *Phacochoerus africanus*) and soft ticks of the *Ornithodoros moubata* complex inhabiting warthog burrows, (2) a tick to domestic pig cycle characterized by the transmission of the virus to domestic pigs by ticks dropped to domestic pig shelters by warthogs, (3) a domestic cycle accounting for most of ASF outbreaks globally where the virus is transmitted by direct contact between infected and susceptible domestic pigs or from infected domestic pig products to domestic pigs, and (4) a wild boar-habitat cycle specific to Eurasian countries [5,8,27]. The sylvatic ASFV cycle specific to eastern and southern Africa is believed to play an important role in the genetic variation of the ASFV and in hindering ASF control [20]. African swine fever outbreaks in domestic pigs are mandated to be reported to the World Organization for Animal Health (OIE) and despite the perceived increase of the reported ASF outbreaks [10], our understanding of the process of ASFV maintenance and spread in eastern and southern Africa is quite limited. The aim of this manuscript was to review systematically the available literature on molecular epidemiology of ASF in Tanzania and neighboring countries in order to establish the transmission dynamics of ASFV between these countries. This information will inform the effective application of ASF control measures and will highlight research gaps warranted for further investigation.

2. Materials and Methods

2.1. Search Strategy

The African swine fever outbreaks records were retrieved from OIE, World Animal Health Information System (WAHIS). Using the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [28], a literature search was carried out at Google Scholar, PubMed, Scopus, and CrossRef databases to find relevant information related to molecular epidemiology of ASF in Tanzania and its neighboring countries. The following search string was used: "African swine fever" OR "African swine fever virus" OR "ASF" OR "ASFV" AND ("Tanzania" OR "Burundi" OR "Democratic Republic of the Congo" OR "Kenya" OR "Malawi" OR "Mozambique" OR "Rwanda" OR "Uganda" OR "Zambia"). The search did not include any limit regarding publication date and articles in English were included in the review. Rayyan QCRI [29] was used for preliminary screening of studies. Articles from PubMed were imported directly to Rayyan QCRI, while the Publish or Perish Software [30] was used to import articles from other databases. A first screening of titles and abstracts was performed, then full text of articles identified as possibly relevant were reviewed. The bibliographies of included articles were assessed for further eligible publications.

2.2. Inclusion and Exclusion Criteria

Two independent reviewers (JNH and CY) electronically searched for studies and screened them according to eligibility criteria, the last search was done on 14th October 2020. The focus was on the articles published in English that describe molecular epidemiology of ASFV in Burundi, Democratic Republic of the Congo (DRC), Kenya, Malawi, Mozambique, Rwanda, Tanzania, Uganda, and Zambia without limit regarding publication date. Abstracts without available full text, articles in language other than English, review articles, duplicated information, and studies describing only ASF diagnosis without sequencing and phylogenetic analysis in order to identify the ASFV genotype were excluded.

2.3. Data Extraction and Analysis

Relevant data from all articles included in the review were extracted and entered in a Microsoft Excel database for further handling. The following data were summarized: authors' name, year of publication, host species, nature of sample (blood, tissue, etc.), genes sequenced, sequences accession numbers, country of the study setting, town or the district of the study (where available), and summary of the findings. Primary authors were contacted in case some data were missing or unclear. The results were described using figures and tables to depict the trend of ASF occurrence over time and space.

2.4. Phylogeographic Analysis

In total, 126 ASFV p72 nucleotide sequences were downloaded from the NCBI GenBank database comprising ASFV strains from Burundi (n = 3), DRC (n = 22), Kenya (n = 17), Malawi (n = 17), Mozambique (n = 14), Tanzania (n = 15), Uganda (n = 11), and Zambia (n = 27). No sequences were found from Rwanda. The included sequences were collected from 1954 to 2019 and they were aligned by CrustalW using MEGA X [31] and subsequently edited using SeaView version 4 [32]. The resulting alignment consisted of 126 sequences (409 nucleotides long). The Smart Model Selection in PhyML (SMS) version 1.8.4 was used to evaluate the best fitting nucleotide substitution model using Akaike information criterion (AIC) as a model selection criterion [33]. The best fitting model for sequences used in this study was the GTR + G (general time reversible model with gamma distributed among-site rate heterogeneity). Therefore, a maximum likelihood phylogenetic tree was reconstructed under the GTR + G model with a bootstrap frequency of 1000 replicates as implemented by MEGA X [31]. TempEst version 1.5.3 [34] was used for temporal signal investigation in the dated-tip tree for further phylogenetic molecular clock

analysis. The sequence data were annotated with year of the collection as stated in the corresponding original paper and the country where samples were collected. The least square dating (LSD) was used for ancestral events dating and rooting the phylogenetic tree based on dates [35] using strict molecular clock. The phylogeography of ASFV was reconstructed from the time-scaled tree generated by LSD and location annotations using PastML with maximum likelihood marginal posterior probabilities approximation (MPPA) and Felsenstein 1981 (F81) model options [36]. The PastML generated tree was visualized and edited using iTOL [37].

3. Results

3.1. Article Selection

In total, 648 articles were collected during the initial search and 472 were included after elimination of duplicates. After titles and abstracts screening against the eligibility criteria, 299 articles were excluded. The majority of excluded publications reported data from countries not concerned by this review and review papers. Therefore, 173 full-text manuscripts were assessed in detail and 34 articles were retained for qualitative synthesis and 29 papers for further molecular assessment (Figure 1). The earliest study meeting the eligibility criteria was Lubisi et al. published in 2005, and therefore the current review considers literature over a 15 years period from 2005 to 2020.

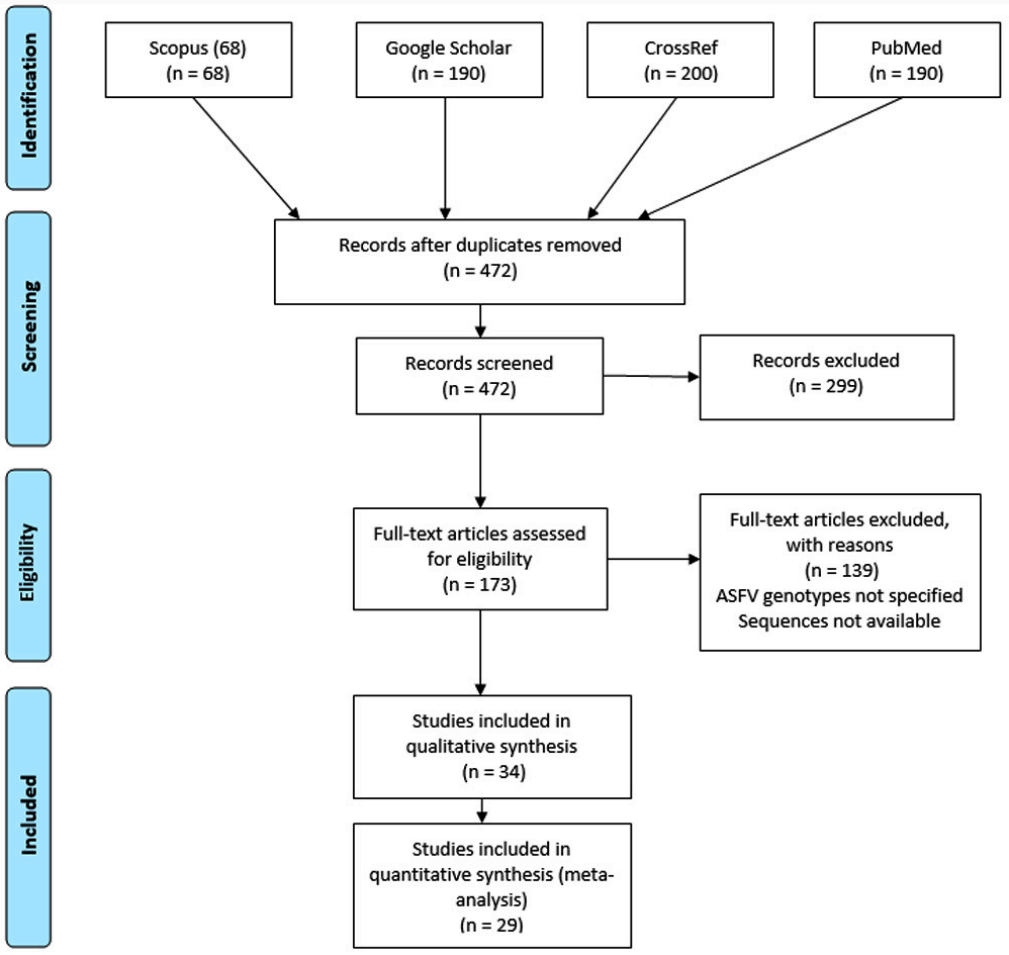

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of the literature search, screening, assessing eligibility, and article selection.

3.2. ASF Disease Pattern

The total of ASF outbreaks reported to OIE by Tanzania and its eight neighboring countries from 2005 to 2019 were 1588 affecting 341,742 cases that led to 302,739 domestic pig deaths during this period. The case fatality rates (CFR) varied from 15.41% to 98.95% with an overall CFR of 88.58%. The number of reported ASF outbreaks, number of cases, number of deaths, and the case fatality rate reported by Tanzania and its eight neighboring countries increased over time from 2005–2009 to 2010–2014 periods (Table 1).

Table 1. African swine fever outbreaks, cases, deaths, and case fatality rate reported to the World Organization for Animal Health (OIE) by Tanzania and its eight neighboring countries from 2005 to 2019.

Time Period	Country	Number of Outbreaks	Number of Cases	Number of Deaths	Case Fatality Rate (%)
2005–2009	Tanzania	5	956	738	77.19
	Rwanda	134	7057	5863	83.08
	Burundi	-	-	-	-
	Malawi	86	16,973	10,785	63.54
	DRC	81	1413	1329	94.05
	Mozambique	78	6715	5194	77.35
	Zambia	43	1570	1271	80.95
	Kenya	9	924	549	59.41
	Uganda	3	401	181	45.13
Subtotal		439	36,009	25,910	71.95
2010–2014	Tanzania	41	4957	4275	86.24
	Rwanda	200	3553	1068	30.06
	Burundi	1	159	26	16.35
	Malawi	139	80,437	77,896	96.84
	DRC	191	153,692	140,493	91.41
	Mozambique	42	3136	2391	76.24
	Zambia	44	3835	2381	62.08
	Kenya	6	203	167	82.26
	Uganda	10	622	473	76.04
Subtotal		674	250,594	229,170	91.45
2015–2019	Tanzania	43	4981	3067	61.57
	Rwanda	47	593	532	89.71
	Burundi	28	3633	560	15.41
	Malawi	19	1813	1666	91.89
	DRC	237	35,407	35,038	98.95
	Mozambique	38	1239	936	75.54
	Zambia	42	5966	5025	84.23
	Kenya	3	231	223	96.53
	Uganda	18	1276	612	47.96
Subtotal		475	55,139	47,659	86.43
Grand total		1588	341,742	302,739	88.58

3.3. ASFV Genotypes

The included studies reported 15 different p72 ASFV genotypes in Tanzania and its eight neighboring countries. The following genotypes were reported: I, II, V, VI, VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XX, and XXIV (Tables 2 and 3). Genotypes V and X were found in domestic pigs, warthogs and ticks; genotype I in domestic pig, ticks and bush pig; genotype IX in domestic pigs and warthogs; genotypes II, VIII, XII, XV, and XIV in both domestic pigs and ticks; while genotypes VI, and XVI were exclusively described in domestic pigs, and genotypes XI, XIII, and XXIV exclusively in soft ticks (Figure 2). Zambia recorded a wider variety of ASFV p72 genotypes with seven genotypes being reported to circulate in the country, namely genotypes I, II, VIII, XI, XII, XIII, and XIV.

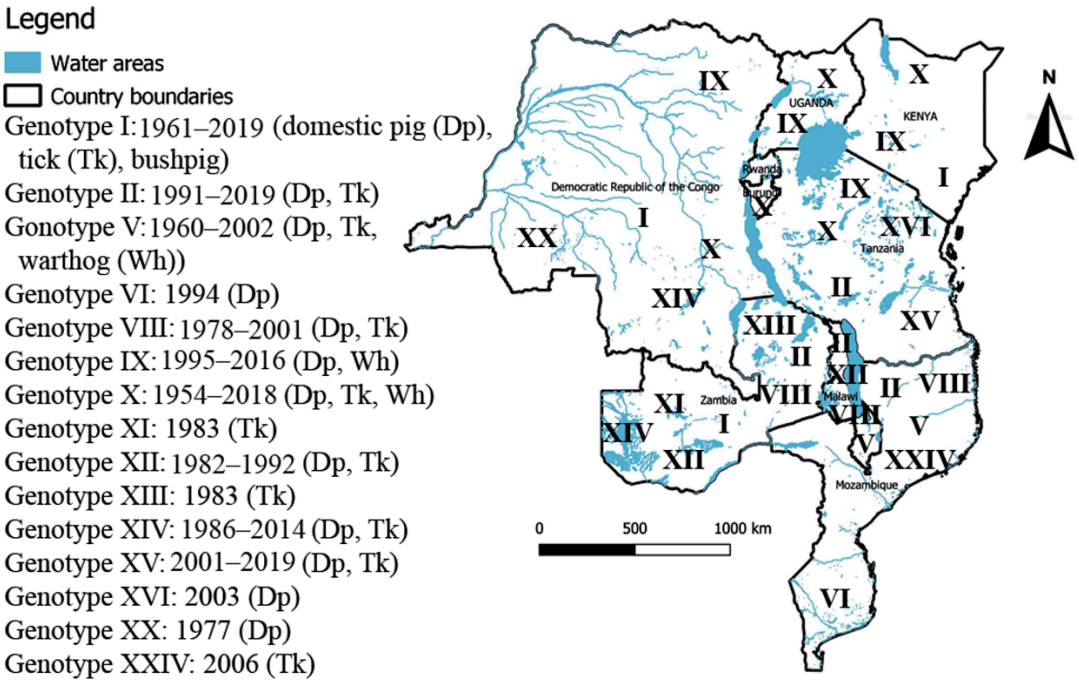

Figure 2. Distribution of the ASFV p72 genotypes circulating between Tanzania and its eight neighboring countries by October 2020. The map was developed using QGIS version 3.4.4 (https://www.qgis.org/en/site/about/index.html).

Table 2. Summary of the ASFV p72 genotypes distribution in Tanzania and neighboring countries. The black highlight indicates that the corresponding genotype is present.

	ASFV p72 Genotype															
Country	**I**	**II**	**V**	**VI**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**	**XIV**	**XV**	**XVI**	**XX**	**XXIV**	**Total Genotypes**
Burundi							■									1
DRC	■					■	■				■			■		5
Kenya	■					■	■									3
Malawi		■	■		■				■							4
Mozambique		■	■	■	■										■	5
Rwanda																0
Tanzania		■				■	■					■	■			5
Uganda						■	■									2
Zambia	■	■			■			■	■	■	■					7
Total countries	3	4	2	1	3	4	5	1	2	1	2	1	1	1	1	

Table 3. African swine fever virus p72 genotypes circulating between Tanzania and its eight neighboring countries by October 2020.

Reference	Targeted Genomic Region	Reported ASFV p72 Genotype	Host Species	Country (Number of Papers)
[38]	p72	I	Tick	Zambia (7)
[39]	Whole genome sequencing	I	Tick	
[40]	p72, p54, p30, CVR	II	Domestic pig	
[41]	p72, p54	I	Domestic pig	
[22]	p72	I, II, VIII, XI, XII, XIII, XIV	Domestic pig and tick	
[23]	p72, CVR	I, VIII	Tick and domestic pig	
[17]	p72, p54, CVR	I, II, XIV	Domestic pig	
[42]	WGS	IX	Domestic pig	Uganda (10)
[43]	p72, p54, CVR	IX	Domestic pig	
[44]	p72, p54, CVR	IX	Domestic pig	
[45]	p72, p54, CVR	IX	Domestic pig	

[46]	WGS	IX	Domestic pig	
[47]	p72, p54, CVR	IX	Domestic pig	
[21]	p72, p54, CVR	IX, X	Domestic pig	
[22]	p72	IX	Domestic pig	
[23]	p72, CVR	IX, X	Domestic pig	
[48]	p72, p54, CVR, TK	IX	Domestic pig	
[49]	p72, CVR	II, V, VI, VIII	Domestic pig	
[21]	p72, p54, CVR	V	Domestic pig	
[22]	p72	II, VIII	Domestic pig	Mozambique (5)
[23]	p72, CVR	VI	Domestic pig	
[20]	p72, CVR, p30, p54	II, V, XXIV	Tick	
[50]	p72, p54	IX	Domestic pig	
[51]	p72, p54, CVR, CD2v, I73R-I329L	X	Domestic pig	
[52]	Whole genome sequencing	XX	Domestic pig	DRC (6)
[22]	p72	I	Not known	
[53]	p72, p54, CVR	I, IX, XIV	Domestic pig	
[23]	p72, CVR	I	Domestic pig	
[54]	WGS	IX, X	Domestic pig	
[15]	WGS	X	Domestic pig	
[16]	p72, p54, p30, CVR	IX, X	Domestic pig, tick and warthog	
[21]	p72, p54, CVR	IX	Domestic pig	Kenya (8)
[48]	p72, p54, CVR, TK	IX	Domestic pig	
[55]	p72, p54	IX	Domestic pig	
[22]	p72	I, X	Warthog, domestic pig and bush pig	
[23]	p72, CVR	X	Warthog	
[56]	p72, p54, CVR, I73R-I329L	II, IX	Domestic pig	
[57]	p72, p54, CVR	XV	Tick	
[58]	p72, p54, CVR	XV	Domestic pig	
[59]	p72	II	Domestic pig	
[60]	p72, p54, CVR	X	Domestic pig	Tanzania (9)
[61]	p72	X	Domestic pig	
[22]	p72	X, XV, XVI	Tick, warthog and domestic pig	
[23]	p72, CVR	X	Warthog	
[62]	p72	II, IX, X	Domestic pig	
[63]	p72, CVR, I73R-I329L	II	Domestic pig	
[21]	p72, p54, CVR	V, VIII	Domestic pig and tick	Malawi (4)
[22]	p72	VIII, V, XII	Domestic pig	
[23]	p72, CVR	V, VIII	Warthog and domestic pig	
[22]	p72	X	Domestic pig	
[23]	p72, CVR	X	Domestic pig	Burundi (3)
[64]	p72, CVR, I73R-I329L	X	Domestic pig	
-	-	-	-	Rwanda (0)

3.4. Phylogeography of ASFV

Results for root to tip divergence showed that the dataset used in this study had a positive temporal signal with the correlation coefficient of 0.19 and R^2 of 0.038. The time to the most recent common ancestor (TMRCA) for ASFV strains circulating between Tanzania and its neighboring countries dated back to 1652.233 (1626.473, 1667.735) with an evolutionary rate of 4.805×10^{-5} (2.5857×10^{-5}, 9.7789×10^{-5}). The location of the root was

unresolved with an indication that Kenya might be the root location with 14.95% probability. Phylogeographic dispersal of ASFV revealed several transboundary spread events from Kenya to Uganda, Tanzania, and Mozambique. From Mozambique, ASFV further spread to Zambia and Malawi and from there to Tanzania. Transmissions from Uganda to DRC, from Tanzania to Burundi were also observed (Figure 3).

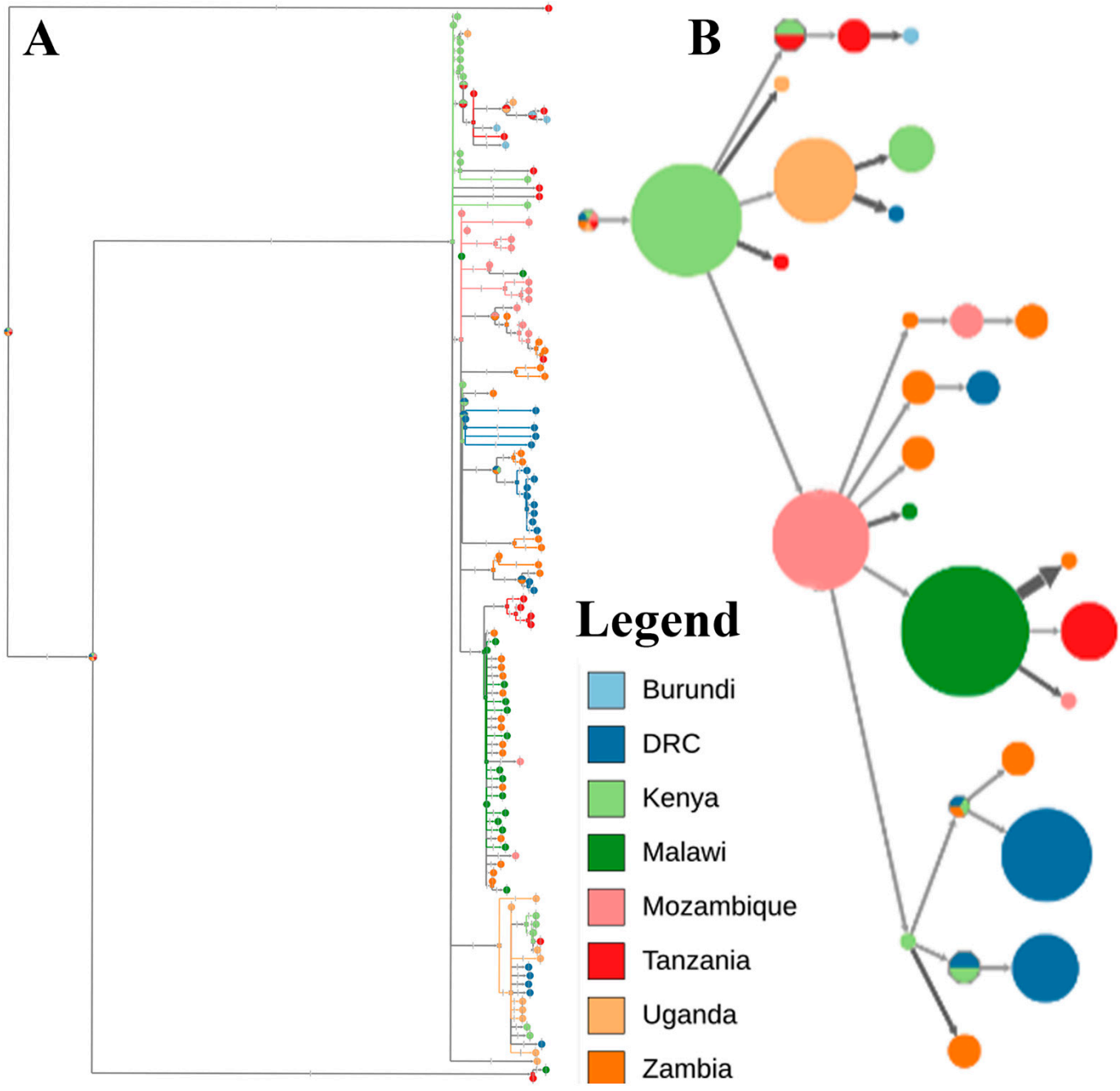

Figure 3. Ancestral reconstruction of African swine fever virus strains collected in Tanzania and its neighboring countries. The figure shows the full tree (**A**) and compressed (**B**) visualizations produced by PastML using MPPA with an F81-like model. Different colors correspond to different countries as shown in the legend.

4. Discussion

This review aimed at investigating the molecular epidemiology of the ASFV strains circulating in Burundi, DRC, Kenya, Malawi, Mozambique, Rwanda, Tanzania, Uganda, and Zambia. Data and articles describing molecular epidemiology of ASF were retrieved from public databases and 34 articles met our inclusion criteria. Our findings suggested a tendency toward the increase of number of reported ASF outbreaks, number of cases, number of deaths, and the case fatality rate over time. A high ASFV genotypic diversity was reported by the included studies with 15 different ASFV genotypes. Additionally, phylogeographic dispersal analysis revealed several transboundary spread events of ASFV strains between countries concerned by this review.

All nine countries concerned by this review reported 1588 ASF outbreaks to the OIE WAHIS from 2005 to 2019 [10]. The real number of ASF outbreaks that occurred in these countries during the same period may be higher than those reported to OIE because the ASF outbreaks reporting in Africa was characterized as sporadic and incomplete for several reasons including poor communication channels and fear of undesirable consequences [4,5]. The increasing trend of reported ASF outbreaks, cases and deaths of domestic pigs could be attributed to the increasing number of ASF outbreaks within the region, with DRC and Malawi reporting 153,692 and 80,437 ASF cases that led to 140,493 and 77,896 domestic pig deaths, respectively, between 2010 and 2014 [10]. In addition, there has been an improvement in laboratory diagnosis capacity and enhanced surveillance systems in order to prevent ASF outbreaks in concerned countries. However, this increase of reported ASF outbreaks is worrisome and regional control programs need to be put in place to control the occurrence of ASF in the region. For some countries (Burundi, Kenya, Malawi, Rwanda, and Zambia), the data for 2019 are not complete because at the time of data retrieving, their 2019 reports were not available on the WAHIS database.

This review showed that 15 of the 24 ASFV p72 genotypes have occurred in Burundi, DRC, Kenya, Malawi, Mozambique, Tanzania, Uganda, and Zambia. These countries are rich in wildlife protected areas where ASFV natural reservoirs exist, making the epidemiology of the ASFV in these countries complex. For instance, the included studies reported diverse range of ASFV p72 genotypes recovered from domestic pigs, warthogs, ticks, and bush pigs in samples collected from 1954 to 2019. Genotypes V and X were isolated from domestic pigs, warthogs and ticks suggesting the association of these genotypes to the ASFV sylvatic cycle as previously described [16,20,54]. The ASFV p72 genotype V was reported to circulate in Malawi and Mozambique since 1960 and the sylvatic cycle was cited to play an important role in its maintenance in those countries [20,49]. The genotype X was recovered from domestic pigs in 1950 in Kenya [15], since then, it was regularly reported to circulate and cause ASF outbreaks in Kenya [15,16,21,23], Uganda [21,23], Tanzania [22,23,61,62], Burundi [22,23,64], and DRC [51]. Recent molecular studies have reported high genetic similarity at three genomic regions of ASFV (p72, CVR and *I73R-I329L*) between the ASFV p72 genotype X strains responsible for ASF outbreaks in 2016 in Kagera region in Tanzania [62,64], the 2018 ASF outbreak in Rutana region in Burundi [64], and the ASF outbreak that occurred during December 2018 to January 2019 in South Kivu province of the DRC [51]. Commercial traffic and cross-border movements of domestic pigs and pork products were speculated as the main drivers of the ASFV spread between DRC, Burundi, and Tanzania [51,53,64]. Rutana region shares a border with Kagera region which borders South Kivu province through Lake Tanganyika and uncontrolled transboundary movements of domestic pigs and pork products are likely to happen in the area. However, the area is rich in wildlife protected areas with natural reservoir of ASFV (mainly warthogs) and their role in the epidemiology of the disease in the area has not been investigated. It would be interesting to investigate the sylvatic cycle of the ASFV in the area in order to get more insight into the epidemiology of ASFV. The included studies reported the ASFV p72 genotype I isolated from domestic pigs, ticks and bush pigs. Genotype I was described as the most prevalent in DRC where it was regularly recovered from domestic pigs since 1963 [22,23,53] and it is described as the most widely

spread ASFV genotype in Zambia where it was isolated from soft ticks and domestic pigs [38,40,41]. Additionally, one study has described the ASFV p72 genotype I isolated from bush pig (*Potamochoerus porcus*) in 1961 in Kenya [22]. Apart from eastern and southern Africa, the ASFV p72 genotype I strains have a wide range of geographical distribution, they have been described in Europe, South America, the Caribbean, and West Africa [22].

The ASFV p72 genotype IX was recovered from domestic pigs and warthogs. This genotype is described as the most predominant in Uganda and Kenya [42], but also, it was described in DRC [50,53] and Tanzania [56,62]. Although associated with lethal ASF outbreaks [21,47,48], an increasing number of studies reported the genotype IX in asymptomatic domestic pigs in Tanzania [56], DRC [50], Uganda [44], and Kenya [65]. The reason behind this variation in virulence of the ASFV p72 genotype IX is worth investigating. The high genetic similarity between the ASFV p72 genotype IX strains recovered from warthogs in central Kenya in 2008 and 2009 [16] to ASFV strains described in domestic pigs in Kenya, Uganda, DRC, and Tanzania highlights the role of the ASFV sylvatic cycle in the epidemiology of the ASFV in eastern Africa.

The included studies reported ASFV p72 genotypes II, VIII, XII, XIV, and XV in both domestic pigs and ticks. The ASFV p72 genotype II was recovered from domestic pigs in Zambia and Mozambique about 30 years ago [22,49] and since then it has expended its geographical distribution not only to eastern and southern Africa countries but also to Madagascar [66], Mauritius [67], Europe [24], and Asia [11,25]. The recent detection of this genotype II in soft ticks in Mozambique provides evidence of a possible sylvatic source of this ASFV p72 genotype [20]. In addition, homology between ASFV strains from ticks collected at pig shelters and those from warthog burrows was observed, suggesting the possibility of ASFV transmission at the wild and domestic pigs interface [20]. Among countries included in this review, the ASFV p72 genotype II was described in Zambia [17,40], Mozambique [20,22,49], Tanzania [56,59,62], and in Malawi [63]. In 2007, the ASFV p72 genotype II escaped from its African geographical distribution to Georgia and subsequently spread to countries of the European Union and Russia before it reached Asia in August 2018 [13,25]. Additionally, the transboundary spread of the highly virulent ASFV p72 genotype II similar to the Georgia 2007/1 strain is highlighted by the introduction of this genotype into Tanzania probably from Malawi [59,62,63] and the emergence of this ASFV genotype in Zimbabwe in 2015 after several years of ASF absence probably from the neighboring Mozambique [68]. Furthermore, the genotype VIII was found to circulate in Zambia, Mozambique and Malawi [21–23], genotype XII was described in Zambia and Malawi [22], whilst genotype XV seems to be confined to Tanzania where both domestic and sylvatic cycles have been described for this genotype [22,57,58]. Additionally, genotype XIV which was previously restricted to Zambia where it was isolated from soft ticks in 1986 [22] was reported in domestic pigs in Zambia [17] and in DRC [53].

Genotypes VI and XVI were described as domestic pig cycle associated genotypes being confined to Mozambique [23,49] and Tanzania [22], respectively, while genotypes XI and XIII were recovered from soft ticks in Zambia in 1983 [22], and the recently identified genotype XXIV was isolated from soft ticks collected in 2006 from Gorongosa Park in Mozambique [20].

High genotypic variability was reported by included studies with 15 different ASFV genotypes being reported to circulate in countries concerned by this review. Movement of domestic pigs and pork products were cited as the main factor of ASFV spreading within and between countries, for instance the ASFV spread pattern was linked to trade highways in Tanzania [62] and Uganda [47] highlighting the importance of the anthropogenic factors and the ASFV domestic pig cycle in the spread of ASF in concerned countries. In addition, the ASFV sylvatic cycle seems to play a role in the maintenance of ASFV in concerned countries as 13 among the 15 circulating genotypes were isolated either from warthogs or ticks. However, the role of bush pigs in the epidemiology of ASF in the countries concerned by this review appears extremely limited as only one study reported ASFV genotype I recovered from bush pig.

This study identified substantial viral dispersal and spread routes between Tanzania and its neighboring countries. The estimated evolution rate of 4.805×10^{-5} substitution/site/year for ASFV strains collected in Tanzania and its eight neighboring countries from 1954 to 2019 is higher than other double-stranded DNA viruses, however, it is similar to the substitution rate of the rapidly evolving RNA viruses [69]. These findings are in agreement with previous studies that estimated the evolution rate for the ASFV strains [70,71]. Our estimated temporal most recent common ancestor (TMRCA) dated back to 1652.233 (1626.473, 1667.735) supporting the hypothesis that the ASFV could have been circulating in Eastern Africa before it was isolated and described for the first time as previously described [70,71]. Phylogeographical dispersal of the ASFV revealed several transboundary spread events from Kenya to Uganda, Tanzania and Mozambique. From Mozambique, ASFV further spread to Zambia and Malawi and from there to Tanzania. Transmissions from Uganda to DRC, from Tanzania to Burundi were also observed as shown in Figure 3. These observations are consistent with studies using traditional phylogenetic methods where transboundary spread of ASFV between DRC and Uganda [50,53], Tanzania and Malawi [59,63], DRC and Zambia [53], Kenya and Uganda [21] were speculated. Our findings highlight the role of neighboring countries in the epidemiology of ASFV and a regional approach would be more effective for the control of ASF. Whole genome sequences of ASFV from the countries concerned by this review are still limited and those data are needed for estimating more accurately the transmission dynamics of the ASFV in these countries and designing an effective control strategy.

5. Conclusions

In conclusion, a considerable diversity of ASFV genotypes were found to circulate in Tanzania and its neighboring countries. Furthermore, a transboundary spread of ASFV between countries was observed. These results suggest persistence of ASFV in these countries and advocate for more research on whole genome sequencing of ASFV and the ASFV sylvatic cycle to improve our understanding of the transmission dynamics of the virus and for a regional approach to mitigate the spread of ASFV.

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Paper Two

Genetic profile of African swine fever virus responsible for the 2019 outbreak in northern Malawi

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Genetic profile of African swine fever virus responsible for the 2019 outbreak in northern Malawi

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Abstract

Background: African swine fever (ASF) is an infectious transboundary animal disease which causes high mortality, approaching 100% in domestic pigs and it is currently considered as the most serious constraint to domestic pig industry and food security globally. Despite regular ASF outbreaks within Malawi, few studies have genetically characterized the causative ASF virus (ASFV). This study aimed at genetic characterization of ASFV responsible for the 2019 outbreak in northern Malawi. The disease confirmation was done by polymerase chain reaction (PCR) followed by molecular characterization of the causative ASFV by partial genome sequencing and phylogenetic reconstruction of the *B646L* (p72) gene, nucleotide alignment of the intergenic region (IGR) between *I73R* and *I329L* genes and translation of the central variable region (CVR) coded by *B602L* gene.

Results: All thirteen samples collected during this study in Karonga district in September 2019 were ASFV-positive and after partial genome sequencing and phylogenetic reconstruction of the *B646L* (p72) gene, the viruses clustered into ASFV p72 genotype II. The viruses characterized in this study lacked a GAATATATAG fragment between the *I173R* and the *I329L* genes and were classified as IGR I variants. Furthermore, the tetrameric amino acid repeats within the CVR of the *B602L* gene of the 2019 Malawian ASFV reported in this study had the signature BNDBNDBNAA, 100% similar to ASFV responsible for the 2013 and 2017 ASF outbreaks in Zambia and Tanzania, respectively.

Conclusions: The results of this study confirm an ASF outbreak in Karonga district in northern Malawi in September 2019. The virus was closely related to other p72 genotype II ASFV that caused outbreaks in neighboring eastern and southern African countries, emphasizing the possible regional transboundary transmission of this ASFV genotype. These findings call for a concerted regional and international effort to control the spread of ASF in order to improve nutritional and food security.

Keywords: African swine fever virus, *Asfarviridae*, Domestic pigs, Molecular characterization, Malawi

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Background

African swine fever (ASF) is a highly contagious, deadly hemorrhagic viral disease of domestic pigs and wild boars of all breeds and ages caused by ASF virus (ASFV), a double-stranded DNA arbovirus and the only member of the family *Asfarviridae*, genus *Asfivirus* [1–3]. The virus is transmitted through a sylvatic cycle involving warthogs (*Phacochoerus africanus*) which do not develop clinical disease and soft ticks of the *Ornithodoros moubata* complex inhabiting warthog burrows [4]. This ancient sylvatic cycle specific to eastern and southern Africa occasionally leads to virus spill to domestic pigs through the tick-pig cycle that involves infected soft ticks dropped by warthogs at pig shelters [5]. After the virus is introduced to the domestic pig population, transmission between domestic pig population occurs through the transmission of the virus among domestic pigs or by feeding contaminated pig products to domestic pigs, accounting for the majority of ASF outbreaks globally [6, 7]. Recently, an additional epidemiological cycle characterized by both direct transmission between infected and susceptible Eurasian wild boar (*Sus scrofa*) and indirect transmission through carcasses in the habitat has been described and named the wild boar-habitat cycle [8]. The current geographical distribution of ASF extends across more than 50 countries in Africa, Europe and Asia and among them, 33 are countries in Africa, south of Sahara [9, 10]. The recent spread of ASF within Europe and Asia has substantially increased the global concern regarding the disease and it is considered as the most devastating disease to global domestic pig industry and food security [2].

Depending on the isolate, the ASF viral genome varies in length from about 170 and 193 kilobase pairs (kbp) and encodes between 150 and 167 open reading frames with a conserved central region and variable termini [2]. Although most of the length variations were described to be associated with the gain or loss of copies within multigene families (MGF), smaller length variations are also associated with the number of tandem repeat sequences (TRS) located at loci both within coding and intergenic regions [11]. Sequence analysis of distinct genomic regions of ASFV has proved to be very useful in identifying the origin and transmission pathways of ASF during outbreaks [12]. Based on the ASFV p72 major capsid protein gene (*B646L*), 24 distinct ASFV genotypes (I-XXIV) have been described [13, 14] and analysis of additional genes has shown to provide higher resolution to distinguish between closely related isolates. The central variable region (CVR) within the *B602L* gene has shown to provide more information about relationship between isolates at genotype, country and regional levels [15–18]. Recent studies have demonstrated the value of the tetrameric repeat sequences (TRS) located in the intergenic region between the *I73R* and *I329L* genes in determining the origin and mapping the spread of closely related ASFV isolates [19, 20]. By combining p72, *B602L* (CVR) and TRS, a high level resolution is achieved for viral discrimination despite the existence of many other markers.

In Africa South of Sahara, the existence of all the 24 ASFV p72 genotypes described to date has been demonstrated [21, 22]. Briefly, in West Africa, where there is no evidence of the existence of the ASFV sylvatic cycle, only genotype I has been reported. In Central, Eastern and Southern Africa where three ASFV transmission cycles exist, rich ASFV genotypic variability exists with all 24 (I to XXIV) ASFV genotypes [9, 13, 22–25]. In Malawi, ASF is endemic and several outbreaks have been reported to the World Organization for Animal Health (OIE) in almost all its provinces [9, 10, 22, 26]. For instance, from January 2005 to December 2018, 227 ASF outbreaks which led to 87,063 pig deaths were reported to OIE [10]. Each year, ASF is reported in different parts of the country posing a serious constraint to the development of the domestic pig industry in Malawi. Despite the regular ASF outbreaks in domestic pigs within Malawi, molecular characterization of the causative viruses has been limited, thus the ASF outbreaks patterns and ASFV genotypes mapping in the country are incomplete. Most of ASFV molecular characterization studies from Malawi were carried out more than a decade ago and previously characterized ASFV strains grouped into p72 genotypes V, VIII and XII and all domestic and sylvatic ASFV transmission cycles have been described in the country [16, 18, 26, 27]. The tick-domestic pig and the sylvatic cycles of ASFV transmission involving warthogs and ticks collected from domestic pig shelters and warthogs' burrows have been demonstrated in Malawi [28–30]. Warthogs and bush pigs which are natural reservoirs for ASFV are commonly found in National Parks and Wildlife Reserves of Malawi [28, 29, 31, 32] and may possibly play a role in the epidemiology of ASF in the country. Proper ASF outbreak investigation and continuous molecular characterization of the responsible viral strains provide insight into the transmission dynamics of the virus, differentiation of closely related strains and identification of potential transmission routes during and after outbreaks in order to guide appropriate interventions for an effective control of ASF [33]. This study aimed at confirming and conducting molecular characterization of the ASFV responsible for the 2019 outbreak in Karonga district located in northern Malawi.

Results

Laboratory confirmation of ASF

All collected tissue samples belonging to 13 different domestic pigs from Karonga district included in the present study were positive for ASFV after conducting diagnostic PCR using ASFV-specific primers.

Phylogenetic reconstruction of ASFV targeting *B646L* (*p72*) gene, TRS and CVR

In order to classify viruses characterized in this study among the 24 ASFV p72 known genotypes, the c-terminal end of *B646L* (p72) gene was amplified and sequenced. All sequences obtained in this study have been deposited to the GenBank and given accession numbers (Accession numbers MN755863-MN755874). The ASFV from domestic pigs in Karonga district named MAL/19/Karonga/1–4 had 100% nucleotide identity. The BLASTn of *B646L* (p72) nucleotide sequences of MAL/19/Karonga/1–4 against other ASFV strains available at GenBank showed 100% nucleotide identity with ASFV strains previously described in Tanzania, Zambia, Georgia, China, Vietnam, Estonia, Moldova, Czech Republic, Belgium and Poland. After phylogenetic reconstruction using ASFV strains indicated in Table 1, the MAL/19/Karonga/1–4 clustered together with ASFV belonging to genotype II (Fig. 1).

The analysis of the intergenic region (IGR) between *I73R* and *I329L* genes of the strains that caused ASF outbreak in Karonga district in northern Malawi in 2019 showed 99.41% nucleotide identity with ASFV genotype II strains responsible for the 2017 outbreaks in Morogoro and Pwani regions of Tanzania and 99.16% nucleotide identity with some isolates circulating in Europe and Asia, including the Georgia 2007/1 isolate. The viruses characterized in this study lacked a GAATATATAG fragment between the *I173R* and the *I329L* genes (Fig. 2) and were classified as IGR I variants as previously described [19, 20]. In addition, a similar G to A replacement were observed in ASFV described in this study and those previously described in Tanzania (Fig. 2).

The CVR sequences obtained in this study were translated into amino acids and coded to obtain corresponding signature. The CVR tetrameric repeats of ASFV that caused the outbreak in Karonga district in 2019 included **CADT, NVDT, CASM, CAST** and **CSTS,** corresponding to B, N, D and A codes, respectively. The ASFV characterized in this study showed 10 amino acid tetramers (**BNDBNDBNAA)** that were 100% identical to each other. A similarity search against other ASFV amino acids sequences performed by BLASTp showed 100% amino acids identity to ASFV that caused previous ASF outbreaks in Tanzania, Madagascar, Zambia, Mozambique, Mauritius, Russia and China (Table 2).

Discussion

In this study, we confirmed an ASF outbreak in Karonga district in northern Malawi that occurred during September 2019. Laboratory confirmation was done by PCR and subsequent genetic characterization of partial ASFV genome by phylogenetic reconstruction of the *B646L* (p72) gene, nucleotide alignment of the intergenic region (IGR) between *I73R* and *I329L* genes and amino acid alignment of the *B602L* (CVR) gene. After phylogenetic analysis, the ASFV strains obtained in this study clustered together with viruses belonging to ASFV p72 genotype II. Furthermore, the IGR and CVR signatures of ASFV strains obtained in this study showed high identity with p72 genotype II viruses previously described in Tanzania, Zambia, Mozambique, Zimbabwe, Georgia, China, Vietnam, Estonia, Moldova, Czech Republic, Belgium, Poland and Russia.

The nucleotide sequences of the c-terminal end of *B646L* (p72) gene of the viruses characterized in this study showed 100% nucleotide identity with those previously described in neighboring countries of Tanzania and Zambia. There is a high possibility of transboundary spread of ASFV between Malawi, Tanzania and Zambia since these countries share a common border and the towns of Karonga in Malawi, Kyela in Tanzania and Mbala in Zambia are less than 400 km apart. The ASFV strain that caused an outbreak in Kyela, Tanzania in 2010 had 100% nucleotide identity for the p72 (*B646L*) gene to Malawian ASFV collected from the 2011 outbreak in Karonga (Table 1). It was previously speculated that the introduction of ASFV genotype II, previously not described in Tanzania, occurred by importation of pig products from Karonga in Malawi [35, 36]. Since then, there has been persistent circulation of highly virulent genotype II viruses in the southern highlands of Tanzania, that have devastated nutritional and food security [40]. Since the introduction of genotype II viruses in Tanzania, these viruses have spread northwards within Tanzania causing devastating impact to the domestic pig industry and expanding the geographical range of this ASFV genotype [34, 39, 40]. Illegal transportation of infected pigs and pig products to uninfected areas have been cited to contribute to the spread of ASFV within Tanzania [34]. There is a need for higher control in order to prevent this ASFV genotype II from its northward spread otherwise it may reach other neighboring East African Community countries, such as Rwanda, Burundi, Uganda and Kenya. It is not uncommon for animal viruses to expand their geographical range as observed with peste des petits ruminants [41–43], Tilapia lake virus disease [44] and foot-and-mouth disease [45–47].

The analysis of the IGR between *I73R* and *I329L* genes showed high nucleotide identity with previously

Table 1 African swine fever virus (ASFV) strains circulating in Malawi together with genotype II ASFV previously described in Tanzania, Zambia, Mozambique,Zimbabwe, Georgia and China, sharing high nucleotide identity with strains that caused outbreak in Malawi during September 2019

Isolate	Host species	Year of isolation	Town/district	Country	Accession number	P72 genotype	Reference
MAL 2011/5	Domestic pig	2011	NK[1]	Malawi	KC835275	II	Unpublished
MAL2011 4	Domestic pig	2011	NK	Malawi	JX524217	II	Unpublished
MAL/2011/3	Domestic pig	2011	NK	Malawi	KC662378	II	Unpublished
Mal 2011/01	Domestic pig	2011	NK	Malawi	JX294724	II	Unpublished
MAL/19/Karonga_1	Domestic pig	2019	Karonga district	Malawi	MN755863	II	This study
MAL/19/Karonga_2	Domestic pig	2019	Karonga district	Malawi	MN755864	II	This study
MAL/19/Karonga_3	Domestic pig	2019	Karonga district	Malawi	MN755865	II	This study
MAL/19/Karonga_4	Domestic pig	2019	Karonga district	Malawi	MN755866	II	This study
TAN/12/Iringa	Domestic pig	2012	Iringa	Tanzania	KF834193	II	[34]
TAN/10/Kyela	Domestic pig	2010	Kyela	Tanzania	JX391987	II	[35]
ZAM/13/Mbala	Domestic pig	2013	Mbala	Zambia	LC174750	II	[12]
ZAM/2017/Mbala/1	Domestic pig	2017	Mbala	Zambia	LC322016	II	[36]
ZIM/2015/01	Domestic pig	2015	Mashonaland	Zimbabwe	KX090923	II	[37]
MOZ_5/2006	Soft tick	2006	Gorongosa National Park	Mozambique	KY353984	II	[14]
Georgia 2007/1	Domestic pig	2007	Caucasus Region	Georgia	NC_044959	II	[38]
China 2018/1	Domestic pig	2018	Shenbei	China	MH722357	II	[20]
Tengani	Warthog	1960	Tengani	Malawi	AF301541	V	[16]
MAL/2002/1	Domestic pig	2002	Mpemba Camp	Malawi	AY494553	V	[27]
Malawi/1978	Domestic pig	1978	NK	Malawi	AF270707	VIII	[16]
ZAM/2/84	Domestic pig	1984	NK	Malawi	AF449471	VIII	[16]
Dezda	Domestic pig	1986	Chilikum-Wera, Dedza	Malawi	AF449479	VIII	[16]
NDA/1/90	Domestic pig	1990	Nadula	Malawi	AF449473	VIII	[16]
BAN/911	Domestic pig	1991	Bangula, Lower Shire	Malawi	AY351501	VIII	[27]
DED/891	Domestic pig	1989	Dedza District	Malawi	AY351502	VIII	[27]
DED/911	Domestic pig	1991	Mtenden Campus, Dedza	Malawi	AY351503	VIII	[27]
DOWA	Domestic pig	1986	Moya, Dowa	Malawi	AY351509	VIII	[27]
KAC/912	Domestic pig	1991	Kachendere Seminary	Malawi	AY351504	VIII	[27]
LIL/891	Domestic pig	1989	Lilongwe District	Malawi	AY351505	VIII	[27]
LIL/901	Domestic pig	1990	Kafere diptank, Lilongwe	Malawi	AY351510	VIII	[27]
MCH/891	Domestic pig	1989	Kachebere Seminary	Malawi	AY351506	VIII	[27]
MCH/893	Domestic pig	1989	Lilongwe District	Malawi	AY351507	VIII	[27]
MCHINJI/075	Domestic pig	1987	Mchinji	Malawi	AY351508	VIII	[27]
NGE/921	Domestic pig	1992	Karonga District	Malawi	AY351544	VIII	[27]
SAL/921	Domestic pig	1992	Salima District	Malawi	AY351546	VIII	[27]
SIY91/2	Domestic pig	1991	Sinyala diptank, Lilongwe	Malawi	AY351566	VIII	[27]
THY/901	Domestic pig	1990	Comforzi farm, Thyolo District	Malawi	AY351545	VIII	[27]
Malawi Lil-20/1 (1983)	Tick (pig)	1983	Chalaswa	Malawi	AY261361	VIII	[30]
MZI/921	Domestic pig	1992	Euthini, Mzinda District,	Malawi	AY351543	XII	[27]

[1]Not known

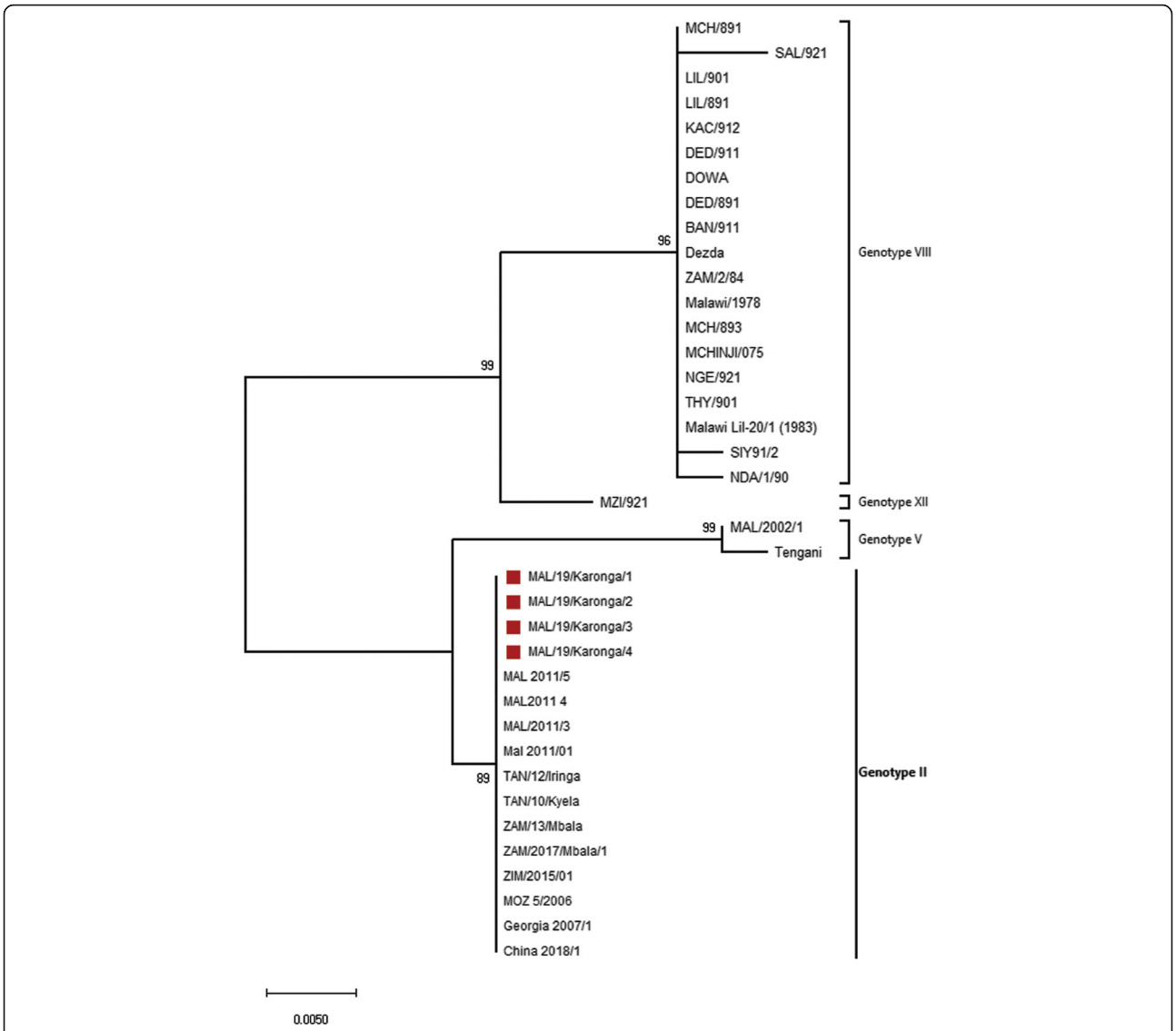

Fig. 1 Maximum Likelihood phylogenetic reconstruction of African swine fever virus (ASFV) strains from Malawi together with genotype II ASFV previously described in Tanzania, Zambia, Mozambique, Zimbabwe, Georgia and China, sharing high nucleotide identity with viruses that caused outbreak in Malawi during September 2019 based on *B646L* (p72) gene nucleotide sequences. Phylogeny was inferred following 1000 bootstrap replications, and the node values show percentage bootstrap support. Scale bar indicates nucleotide substitutions per site. The square indicates the African swine fever virus strains that caused outbreak in Malawi during September 2019

characterized ASFV genotype II strains and lacked a GAATATATAG fragment similar to ASFV strains circulating in Tanzania, different countries of Europe and Asia including the isolate Georgia 2007/1 collected in Georgia in 2007 that subsequently spread to other countries of eastern Europe and China [39, 48, 49]. In addition, we observed a similar G to A replacement in strains characterized in this study to ASFV strains responsible for the 2017 outbreaks in Morogoro and Pwani regions of Tanzania [39]. This suggest that ASFV genotype II strains circulating in Tanzania and Malawi are from probably the same source. Whole genome sequencing of strains described in this study and ASFV circulating in eastern and southern Africa will be able to discriminate closely related strains and establish more accurately epidemiological links between different ASF outbreaks occurring in the region.

The tetrameric amino acids repeats within the CVR of the *B602L* gene of the 2019 Malawian ASFV reported in this study had the signature BNDBNDBNAA which was 100% similar to the ASFV strains that caused the ASF outbreaks in Tanzania in 2017 [39] and during 2013 in Zambia [12]. The ZAM/13/Mbala virus was collected in April 2013 from domestic pigs reared in a village along

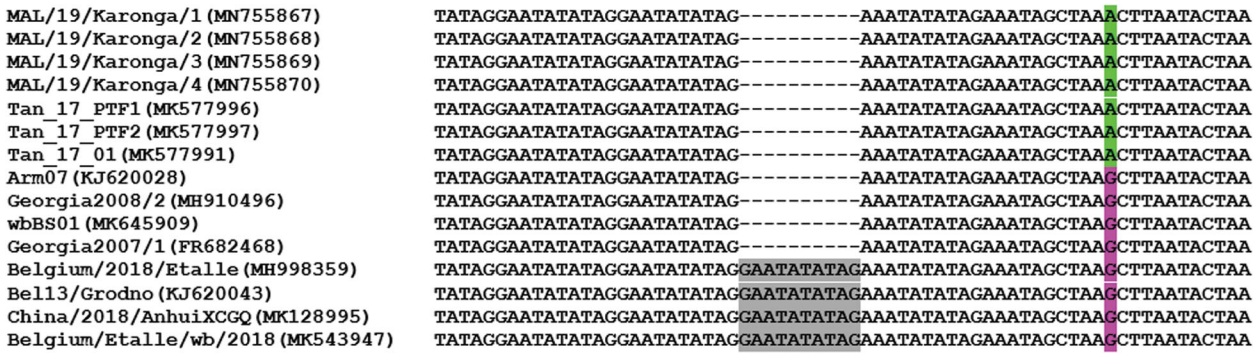

Fig. 2 Nucleotide sequence alignment of the intergenic region between *I73R* and *I329L* genes of African swine fever virus strains belonging to *B646L* (P72) genotype II from Tanzania, Europe and China. The nucleotides highlighted in gray are absent in some viruses including the strains that caused outbreak in Malawi during September 2019. Also, a substitution of G by A is observed in Tanzanian and Malawian viruses only

the border with neighboring Tanzania and a suspected introduction from Tanzania through trans-border trade of pigs and pork products is speculated [12]. Kyela at the Tanzanian side, Karonga in northern Malawi and Mbala in Zambia share borders and ASF outbreaks have been reported to temporally coincide [12, 35, 36].

Comparison of the 3 ASFV genomic regions analyzed in this study revealed high identity between the strains characterized in this study and other viruses belonging to ASFV p72 genotype II that have caused previous outbreaks elsewhere including in Madagascar in 1998 [50]. The first ASFV reported in Madagascar was identical to the virus recovered from the 1994 ASF outbreak in Mozambique and was suspected to be the most likely source of ASF infection in Madagascar that was previously free from ASF [50, 51]. The ASFV p72

Table 2 Selected African swine fever viruses belonging to p72 genotype II with high amino acids sequences identity with viruses collected in Malawi in September 2019 at the Central variable region (CVR) of the *B604L* gene

Virus name	Year	Country of origin	Host species	CVR Accession number	CVR signature	reference
MAL/19/Karonga_1	2019	Malawi	Domestic pig	MN755871	BNDBNDBNAA	This study
MAL/19/Karonga_2	2019	Malawi	Domestic pig	MN755872	BNDBNDBNAA	This study
MAL/19/Karonga_3	2019	Malawi	Domestic pig	MN755873	BNDBNDBNAA	This study
MAL/19/Karonga_4	2019	Malawi	Domestic pig	MN755874	BNDBNDBNAA	This study
ASFV_Tan_17_PTF2	2017	Tanzania	Domestic pig	MK276893	BNDBNDBNAA	[39]
ASFV_Tan_17_PTF1	2017	Tanzania	Domestic pig	MK276892	BNDBNDBNAA	[39]
ASFV_Tan_17_01	2017	Tanzania	Domestic pig	MK276887	BNDBNDBNAA	[39]
ASFV_Tan_15_4	2015	Tanzania	Domestic pig	MK276894	BNDBNDBNAA	[39]
ZAM/13/Mbala	2013	Zambia	Domestic pig	BAW94569	BNDBNDBNAA	[12]
ZAM/2017/Mbala/1	2017	Zambia	Domestic pig	LC322013	BNDBNDBNAA	[36]
Antani03	NK[1]	Madagascar	Domestic pig	EU649696	NK	Unpublished
Arm07	2007	Armenia	Domestic pig	JX857522	NK	[19]
MOZ_2/2006	2006	Mozambique	Tick	ATD84005	BNDBNDBNAA	[14]
MOZ/1/2002	2002	Mozambique	Domestic pig	QBG64414	NK	Unpublished
CN201801	2018	China	Domestic pig	AYD60223	NK	Unpublished
ASFV-wbBS01	2018	China	Wild boar	QAU54736	NK	Unpublished
MAD/1998	1998	Madagascar	Domestic pig	AAQ18412	NK	[16]
Tver0511/Torjo	2011	Russia	Domestic pig	AII03124	NK	[19]
Irkutsk2017	2017	Russia	Domestic pig	AUC64211	NK	Unpublished
MAU/1/2008	2008	Mauritius	Domestic pig	QBG64413	NK	Unpublished

Key: (CAST, CVST, CTST, CASI = A), (CADT, CADI, CTDT, CAGT, CVDT = B), (NVDT, NVGT, NVDI=N) and (CASM = D)
[1]Not known

genotype II was recovered from domestic pigs in the year 2002 and from soft ticks in 2006 in Mozambique [14]. In 2007, the ASFV p72 genotype II strain with high identity to viruses previously described in Madagascar and Mozambique was reported in Mauritius for the first time and swill feeding to domestic pigs was suspected to be responsible for the introduction of the ASFV to the island [52]. The ASFV genotype II occurred in the Caucasus region of Georgia in 2007 with subsequent spread to Russia, different countries of Europe before it reached China in August 2018 and spread to neighboring Asian countries [19, 20, 49]. The virus responsible for the ASF outbreak in Georgia in 2007 was closely related to ASFV strains previously described in Mozambique, Zambia and Madagascar, thus the southern Africa countries or Madagascar were suspected to be the most likely source of ASF infection of the Georgia 2007 ASF outbreak [49]. Eastern and southern Africa countries including Malawi are characterized by the presence of wildlife protected areas with warthogs and ticks of the *Ornithodoros moubata* complex inhabiting warthogs' burrows. These natural reservoir of the ASFV play an important role in the maintenance and transmission of the ASFV through the sylvatic cycle of the virus [14, 22, 53]. The 2015 ASF outbreak in Zimbabwe was caused by the ASFV genotype II after several years without ASF outbreak reported in the country and the transboundary spread from neighboring Mozambique was suspected [37]. The high identity between ASFV strains described in this study and viruses previously characterized in southern Africa countries, Madagascar, Europe and Asia suggests that they may have probably the same wild source and maintained through domestic cycle. In Malawi, the sylvatic cycle of ASFV involving ticks collected from warthogs habitat has been previously described [29] and a detailed study need to be carried out to assess the current role of wild suids and *Ornithodoros* ticks in the maintenance and transmission of ASFV in Malawi.

Conclusions

The virus responsible for the 2019 ASF outbreak in Karonga district clustered into p72 genotype II and showed high nucleotide identity with ASFV strains causing outbreaks in neighboring eastern and southern Africa countries suggesting that the same ASFV strains are causing outbreaks across borders. In addition, ASFV strains described in this study were closely related to viruses previously reported in Europe and Asia. These findings highlight the need for a concerted regional and international effort to control the spread of ASF in order to improve nutritional and food security. Investigation of the role of ASFV sylvatic cycle and further characterizations by whole genome sequencing are needed to fully understand molecular epidemiology of ASFV in Malawi.

Methods

Study area and sample collection

Samples used in this study were collected in Karonga district in northern Malawi from small-scale pig farmers in response to a report from local veterinarians to the Malawian National Veterinary Epidemiology Unit of a hemorrhagic disease affecting many domestic pigs with clinical symptoms suggestive of ASF in September 2019 (Fig. 3). Two tissue samples per domestic pig including spleen and liver were aseptically collected from thirteen dead pigs and transported to the laboratory. In the laboratory, samples were processed by homogenization in sterile phosphate-buffered saline (PBS) at a ratio of 1: 10 w/v followed by centrifugation at 6000 *g* for 5 min and cryopreservation of the supernatant at − 80 °C until DNA extraction.

DNA extraction and nucleotide amplification

QIAmp nucleic acid extraction kit (Qiagen, Hilden, Germany) was used for DNA extraction from collected samples, following manufacturer's instructions. The presence of ASFV in collected samples was confirmed by polymerase chain reaction (PCR) using ASF diagnostic primers PPA1 and PPA2, as previously described by Aguero et al. [54]. The variable 3′-end of *B646L* gene encoding the major capsid protein p72, the tetramer amino acid repeats within the hypervariable central variable region (CVR) and the intergenic region (IGR) between *I73R* and *I329L* were amplified using the following primers: p72-D/p72-U [16], ORF9L-F/ORF9L-R [55] and ECO1A/ECO1B [19], respectively.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of PCR products were obtained by automated dideoxynucleotide cycle sequencing using BigDye Terminator Cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequence scanner software version 2.0 (Applied Biosystems, Foster City, CA) and Bioedit version 7.2.5 (Ibis Biosciences, Carlsbad, CA) were used to check the quality of raw sequences data and to obtain consensus nucleotide sequences from both forward and reverse primers for each of the amplified regions. The obtained consensus nucleotide sequences were used for BLASTn to search for similarity of nucleotide sequences obtained in this study to other nucleotide sequences available at GenBank. The dataset for p72 phylogenetic reconstruction consisted of 38 nucleotide sequences (402 characters), comprising of 4 sequences generated in this study and 34 homologous sequences from GenBank, including ASFV strains previously described in Malawi and

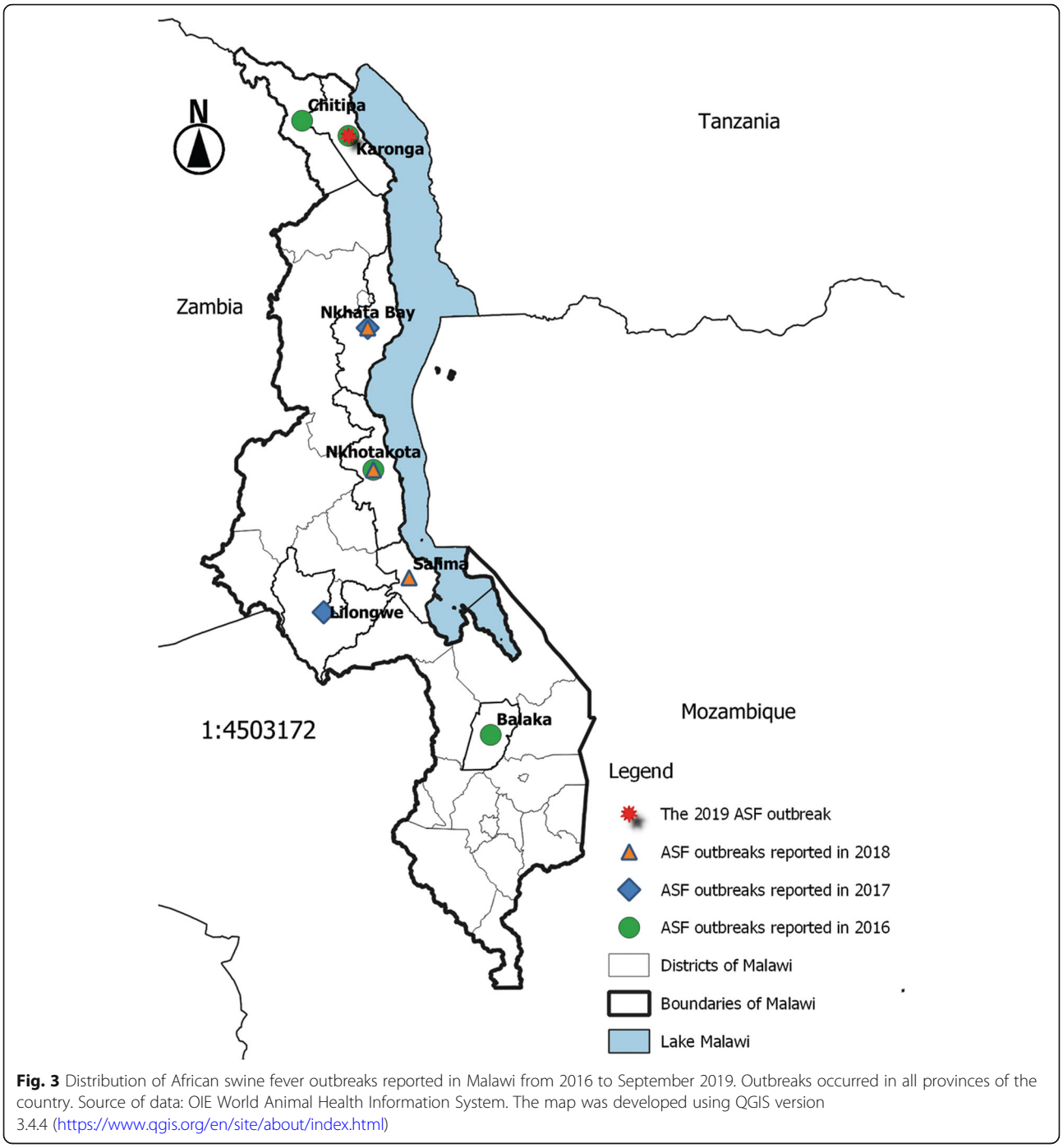

Fig. 3 Distribution of African swine fever outbreaks reported in Malawi from 2016 to September 2019. Outbreaks occurred in all provinces of the country. Source of data: OIE World Animal Health Information System. The map was developed using QGIS version 3.4.4 (https://www.qgis.org/en/site/about/index.html)

genotype II ASFV strains from Tanzania, Zambia, Mozambique, Zimbabwe, Georgia and China, sharing high nucleotide identity with strains described in this study (Table 1). The phylogenetic tree construction was performed using Maximum Likelihood method and Kimura 2-parameter model with a bootstrap frequency of 1000 replicates as implemented by MEGA X [56]. The tandem repeat sequences (TRS) in the intergenic region between *I73R* and *I329L* genes of the strains characterized in this study were compared with other ASFV strains using CrustalW as implemented in MEGA X [56]. The central variable region nucleotide sequences of our ASFV isolates were translated and coded to obtain signatures based on previously reported codes [18, 34, 57]. A similarity search against other ASFV amino acid sequences was performed using BLASTp.

Abbreviations
ASF: African swine fever; ASFV: African swine fever virus; BLASTn: Basic Local Alignment Search Tool for Nucleotides; BLASTp: Basic Local Alignment Search Tool for Proteins; CVR: Central variable region; DNA: Deoxyribonucleic acid; IGR: Intergenic region; MEGA: Molecular Evolutionary Genetics Analysis; OIE: World Organization for Animal Health; P72: African swine fever major capsid protein; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; TRS: Tandem repeat sequences

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Authors' contributions
JNH participated in sample collection, laboratory analysis, data analysis and development of the first draft of the manuscript. GK participated in the study design, sample collection and actively revised the manuscript. GM and JNH conceived the idea, analyzed, interpreted data and revised the manuscript. OK, GM, HJN and JLCC contributed to the conception of the idea, design, interpretation of data and actively revised the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analysed during the current study are available at the GenBank repository (https//ncbi.nlm.nih.gov/genbank) with accession numbers MN755863 to MN755874.

Ethics approval and consent to participate
The samples used in this study were collected from naturally dead domestic pigs according to common veterinary practice as part of routine veterinary investigation by the District Veterinary Officer under the supervision of the national veterinary epidemiologist according to the Malawian Control and Diseases of Animals Act (CAP 66:02 of 1967) and to rule 6 of the Swine Fever Rules G.N. 209/1968. Oral consent was obtained from the domestic pig owners before sampling of their dead domestic pigs and documented in the District Veterinary Officer registry.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Paper Three

Genetic Analysis of African Swine Fever Virus From the 2018 Outbreak in South-Eastern Burundi

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African swine fever (ASF) is a contagious viral disease that causes high mortality, approaching 100%, in domestic pigs and wild boars. The disease has neither a cure nor a vaccine, and it is caused by an ASF virus (ASFV), the only member of the family *Asfarviridae*, genus *Asfivirus*, and the only known DNA arbovirus. Twenty-four genotypes of ASFV have been described to date, and all of them have been described in Africa. ASF is endemic in Burundi, and several outbreaks have been reported in the country; the disease continues to economically impact on small-scale farmers. This study aimed at genetic characterization of ASFV that caused an ASF outbreak in the Rutana region, Burundi, in the year 2018. Tissue samples from domestic pigs that died as a result of a severe hemorrhagic disease were collected in order to confirm the disease using polymerase chain reaction (PCR) and to conduct partial genome sequencing. Nucleotide sequences were obtained for the *B646L* (p72) gene, the intergenic fragment between the *I73R* and *I329L* genes, and the central variable region (CVR) of the *B602L* gene. Phylogenetic analysis of the Burundian 2018 ASFV grouped the virus within *B646L* (p72) genotype X and clustered together with those reported during the 1984 and 1990 outbreaks in Burundi with high nucleotide identity to some ASFV strains previously reported in neighboring East African countries, indicating a regional distribution of this ASFV genotype. Analysis of the intergenic fragment between *I73R* and *I329L* genes showed that the Burundian 2018 ASFV described in this study lacked a 32–base pair (bp) fragment present in the reference genotype X strain, Kenya 1950. In addition, the strain described in this study had the signature AAABNAABA at the CVR (*B602L*) gene and showed 100% amino acid sequence identity to viruses responsible for recent ASF outbreaks in the region. The virus described in this study showed high genetic similarities with ASFV strains previously described in domestic pigs, wild suids, and soft ticks in East African countries, indicating a possible common wild source and continuous circulation in domestic pigs in the region.

Keywords: African swine fever, *Asfarviridae*, Burundi, domestic pigs, genotyping

INTRODUCTION

African swine fever (ASF) is a contagious and fatal viral disease of domestic pigs and wild boar (1, 2). It is caused by the ASF virus (ASFV), the only member of the family *Asfarviridae*, genus *Asfivirus* (3), and the only known DNA arbovirus. Twenty-four (I to XXIV) genotypes of ASFV have been described to date based on nucleotide sequencing of the *B646L* gene encoding for the p72 protein (4–6), and all of them have been described in Africa (2). Depending on the virus strain, the ASFV genomes vary in length from about 170 to 193 kilobase pairs (Kbp) and contain between 151 and 167 open reading frames with a conserved central region and variable termini (7). Depending on the ASFV strain, morbidities and mortalities can reach 100%, making ASF the most serious constraint to domestic pig production, food and nutritional security, and livelihood of small-scale farmers in Africa (8). ASF has neither a cure nor a vaccine, and its effective control relies on quarantine, stamping out, and strict biosecurity measures (9, 10). ASF is endemic in many African countries south of the Sahara and in Sardinia (Italy), and in recent years, it has spread beyond its traditional geographical boundaries to the Caucasus region, the European Union, and Asia (11–14). The recent spread to China, which is the major pork-producing country, is threatening global food security (15, 16). The epidemiology of ASF is complex, transmission is direct and vector-borne, and the disease has well-recognized sylvatic and domestic cycles (17). In Eastern and Southern Africa, ASFV is maintained in a sylvatic cycle between warthogs (*Phacochoerus africanus)* and soft argasid ticks of the *Ornithodoros moubata* complex (18). Warthogs and bushpigs (*Potamochoerus* spp.) are the natural hosts of ASFV that are persistently infected with no obvious clinical disease, and soft ticks of the genus *Ornithodoros* are vectors for transmission of ASFV from the sylvatic to the domestic cycle (19). Wild natural hosts of ASFV have been reported to be present in the Kibira and Ruvubu National Parks of Burundi (20), but their role in the maintenance and transmission of the virus in the country is not known. In the domestic cycle, two transmission patterns are recognized, namely, a tick-to-pig cycle that involves soft ticks inhabiting pig shelters and an exclusively pig-to-pig cycle. Once introduced into domestic pig

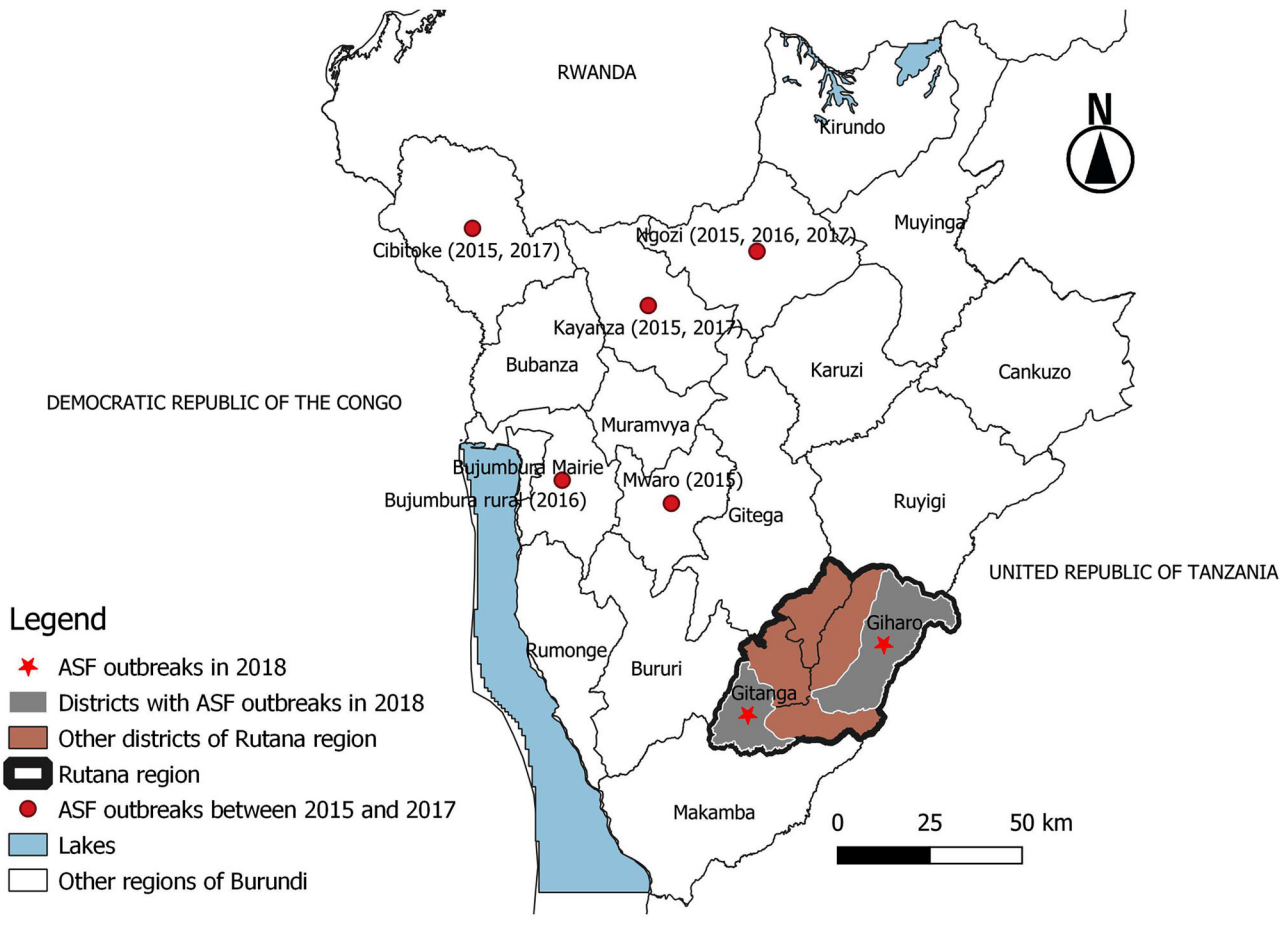

FIGURE 1 | Map of Burundi showing African swine fever outbreaks between 2015 and 2018. The outbreaks between 2015 and 2017 occurred in regions located in the northern part of Burundi bordering Rwanda and western regions bordering the Democratic Republic of the Congo. The 2018 outbreak occurred in the Giharo and Gitanga districts of the Rutana region bordering Tanzania. Source: National Veterinary Laboratory of Burundi, Burundi.

populations, the virus can be transmitted between domestic pigs mainly by ingestion of contaminated feeds and direct contact between infected and susceptible pigs (21).

In Eastern and Southern Africa, some ASFV genotypes are country specific, while others have a transboundary distribution (22). In Burundi, strains of ASFV described from the outbreaks of 1984 and 1990 belong to *B646L* (p72) genotype X (21). Genotype X is one of the predominant genotypes in East African countries including Tanzania, Kenya, and Uganda (23–25). Despite the regular ASF outbreak reports in domestic pigs in Burundi, molecular characterization of the causative viruses has been limited. For instance, the currently available ASFV strains genetically characterized from Burundi were collected more than two decades ago. In August 2018, an outbreak of a hemorrhagic and fatal disease affecting domestic pigs suspected to be ASF occurred in the Rutana region in South-Eastern Burundi. This study describes the confirmation and molecular characterization of the 2018 outbreak of ASFV in South-Eastern Burundi based on partial amplification and nucleotide sequencing of the *B646L* (p72) gene, the tandem repeat sequence (TRS) located between the *I73R* and *I329L* genes, and the central variable region (CVR) of the *B602L* gene.

MATERIALS AND METHODS

Study Area, Sampling, and Sample Processing

An outbreak of a hemorrhagic disease associated with high mortalities in domestic pigs occurred in South-Eastern Burundi

TABLE 1 | African swine fever virus (ASFV) isolates from Eastern and Southern Africa used for the construction of phylogenetic tree based on partial *B646L* (p72) gene nucleotide sequences.

Isolate	Host species	Year of isolation	Location	Country	Accession number	p72 genotype	Reference
DRC/35/10/5	Domestic pig	2010	NK[a]	DRC[b]	KX121552	I	(30)
TAN/12/Iringa	Domestic pig	2012	Iringa	Tanzania	KF834193	II	(31)
BOT/1/99	Domestic pig	1999	NK	Botswana	AF504886	III	(27)
RSA/1/99/W	NK	1999	NK	South Africa	AF449477	IV	(27)
Tengani	Warthog	NK	Tengani	Malawi	AF301541	V	(32)
SPEC265	Domestic pig	1994	NK	Mozambique	AF270710	VI	(32)
RSA/1/98	NK	1998	NK	South Africa	AF302818	VII	(27)
MOZ/1/98	Domestic pig	1998	Tete	Mozambique	AF270705	VIII	(27)
Ug12.Kabale1	Domestic pig	2012	Kabale	Uganda	KC990890	IX	(33)
BUR/18/Rutana	Domestic Pig	2018	Rutana	Burundi	MK829709	X	This study
Kenya 1950	Domestic pig	1950	NK	Kenya	AY261360	X	(34)
TAN/Kwh12	Warthog	1968	Kirawira	Tanzania	AF301546	X	(27)
KAB 94/1	Domestic pig	1994	NK	Kenya	AY972163	X	(35)
KIRT/893	Ticks	1989	Kirawira	Tanzania	AY351512	X	(5)
TAN/16/Ngara	Domestic pig	2016	Ngara	Tanzania	MF437293	X	(36)
TAN/15/Mwanza	Domestic pig	2015	Mwanza	Tanzania	MF437291	X	(36)
BUR/1/84	Domestic pig	1984	Gitega	Burundi	AF449463	X	(27)
BUR/90/1	Domestic pig	1990	Muyinga	Burundi	AF449472	X	(5)
Ken05/Tk1	Tick	2005	Kapiti plains	Kenya	NC_044945	X	(34)
KAB/62	Ticks	1983	Livingstone Game Park	Zambia	AY351522	XI	(5)
MZI/921	Domestic pig	1992	Mzinda	Malawi	AY351543	XII	(5)
SUM/1411	Ticks		Sumbu Park	Zambia	AY351542	XIII	(5)
DRC/35/10/3	Domestic pig	2010	Ngaliema	DRC	KX121550	XIV	(30)
TAN/08/Mazimbu	Domestic pig	2008	Mazimbu	Tanzania	GQ410765	XV	(37)
TAN/2003/1	Domestic pig	2003	Arusha	Tanzania	AY494550	XVI	(5)
ZIM/92/1	Domestic pig	1992	Gweru	Zimbabwe	DQ250119	XVII	(38)
NAM/1/95	NK	1995	Windhoek	Namibia	DQ250122	XVIII	(38)
SPEC/251	NK	1996	Ellisras	South Africa	DQ250118	XIX	(38)
Lillie	Domestic pig	NK	NK	South Africa	DQ250109	XX	(38)
RSA/1/96	NK	1996	Gravelotte	South Africa	DQ250125	XXI	(38)
SPEC/245	NK	NK	Louis Trichardt	South Africa	DQ250117	XXII	(38)
ETH/5a	Domestic pig	2011	Bahir Dar	Ethiopia	KT795361	XXIII	(4)
MOZ_11/2006	Tick	2006	Gorongosa National Park	Mozambique	KY353990	XXIV	(6)

[a] *Not known.*
[b] *Democratic Republic of the Congo.*

in August 2018. The disease started in Mutwana village in the Giharo district of the Rutana region in South-Eastern Burundi (**Figure 1**). The number of domestic pigs that died during the outbreak was recorded from Rutana Region Livestock Office records. Tissues (lung, spleen, and liver) were collected from three domestic pigs that naturally died from the disease. Each tissue (lung, spleen, and liver) was aseptically collected into a separate tube. Samples were chilled on ice and transported to the laboratory. In the laboratory, 1 g from each of the tissue samples was separately placed into a sterile petri dish and chopped using a sterile scalpel blade in the presence of 10 mL sterile phosphate-buffered saline (PBS). Afterward, homogenized tissue samples were centrifuged at 6,000 g for 5 min, and the supernatants, aliquoted into cryovials before cryopreservation at −80°C until DNA extraction.

DNA Extraction

Frozen aliquots of lung, liver, and spleen homogenates were allowed to thaw, and DNA was extracted directly from 150 μL of homogenized tissue samples using a QiaAmp nucleic acid extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Each extraction yielded 50 μL of DNA whose quantity and purity were determined by a nanodrop spectrophotometer (Biochrom, Cambridge, England) before being stored at −20°C until nucleotide amplification by polymerase chain reaction (PCR).

Amplification of ASFV DNA

The disease confirmation was carried out by PCR using ASF diagnostic primers PPA1 and PPA2 as previously described by Agüero et al. (26). Amplification for partial nucleotide sequencing of ASFV DNA was conducted using primers that target (i) the variable 3′-end of the *B646L* gene encoding the major capsid protein p72 using primers p72D and p72U (27), (ii) a TRS located between the *I73R* and *I329L* genes using primers ECO1A and ECO1B (28), and (iii) the CVR of the *B602L* gene using the ORF9L-F and ORF9L-R primer pair (21, 29). The amplification conditions used in the present study were similar to those previously described (21, 26–29). All nucleotide amplifications were performed using AccuPower PCR premix (Bioneer, Daejeon, Republic of Korea) on a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA). Afterward, the electrophoretic separation of amplicons was conducted on 1.5% agarose gel mixed with GelRed nucleic acid stain (Phenix Research Products, Candler, NC) against a 1 Kbp molecular weight marker (Promega, Madison, WI, USA) before visualization and imaging using a Gel Doc^{TM} EZ Imager agarose gel imaging system (Bio-Rad, Hercules, CA).

ASFV Partial Genome Nucleotide Sequencing

PCR products from *B646L* (p72), TRS, and CVR were subjected to automated dideoxynucleotide cycle sequencing using a Big Dye Terminator Cycle sequencing kit V3.1 (Applied Biosystem, Foster City, CA) using primers: p72D, p72U, ECO1A, ECO1B, ORF9L-F, and ORF9L-R. Products from the cycle sequencing reaction were purified by ethanol precipitation and separated by capillary gel electrophoresis on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Chromatograms for both the forward and the reverse primer reactions were checked for quality using Sequence Scanner v2.0 software (Applied Biosystems, Foster City, CA). The forward nucleotide sequence and the reverse complement nucleotide sequence from the reverse primer were subjected to pairwise alignment in Bioedit v7.2.5 (Ibis Biosciences, Carlsbad, CA) in order to obtain a single consensus nucleotide sequence delimited by the forward and reverse primers. In addition to the Burundian 2018 ASFV, the TRS between the *I73R* and *I329L* genes of the Tanzanian ASFV strains TAN/13/Arusha, TAN/16/Babati, and TAN/16/Ngara was amplified and sequenced in this study. The CVR of the *B602L* gene was amplified and sequenced for TAN/16/Ngara in the present study.

Phylogenetic Analysis of ASFV *B646L* (p72), TRS, and CVR

The nucleotide sequences of *B646L* (p72), TRS, and CVR from the 2018 ASFV that caused an outbreak in South-Eastern Burundi were submitted to GenBank and assigned accession

TABLE 2 | Tetramer amino acid repeat signatures within the central variable region (CVR) of the *B604L* gene of selected ASFV strains belonging to p72 genotype X from some East African countries.

Strain name	Year of collection	Country of origin	Host	CVR accession number	CVR signature	Reference
BUR/18/Rutana	2018	Burundi	Domestic pig	MT550685	AAABNAABA	This study
TAN/16/Ngara	2016	Tanzania	Domestic pig	MT550686	AAABNAABA	This study
TAN/13/Arusha	2013	Tanzania	Domestic pig	KF706367	$BNBA(BN)_5NA$	(23)
Ken05/Tk3	2005	Kenya	Tick	HM745290	AAANAABBA	(39)
TAN/13/Moshi	2013	Tanzania	Domestic pig	KF706364	$BNBA(BN)_5NA$	(23)
Ken08BP/HB	2008	Kenya	Bushpig	JN590917	AAABNAAAABA	Unpublished
Bur90/1	1990	Burundi	Domestic pig	AM259424	AAABNAAAAAAAAAABA	(21)
Bur84/2	1984	Burundi	Domestic pig	AM259423	AAABNAAAAAAAAAABA	(21)
Bur84/1	1984	Burundi	Domestic pig	AM259422	AAABNAAAAAAAAAABA	(21)

Key: (CAST, CVST, CTST, CASI = A), (CADT, CADI, CTDT, CAGT, CVDT = B), (NVDT, NVGT, NVDI = N), and (CASM = D).

numbers (**Tables 1**, **2**). The similarity search of the obtained nucleotide sequences against other ASFV sequences available at GenBank was performed using BLASTn (version 2.8.1+). The nucleotide sequence of *B646L* (p72) of the Burundian 2018 ASFV outbreak was aligned with other ASFV nucleotide sequences representing the 24 ASFV *B646L* (p72) genotypes (6, 11, 15) using the ClustalW algorithm in MEGA X (40). ClustalW was used to perform multiple sequence alignment of nucleotide sequences of the TRS as implemented in MEGA X (40). Nucleotide sequences of the *B602L* (CVR) gene were translated using the ExPASy translation tool (https://web.expasy.org/translate/) and coded in order to obtain corresponding amino acid tetramer signatures

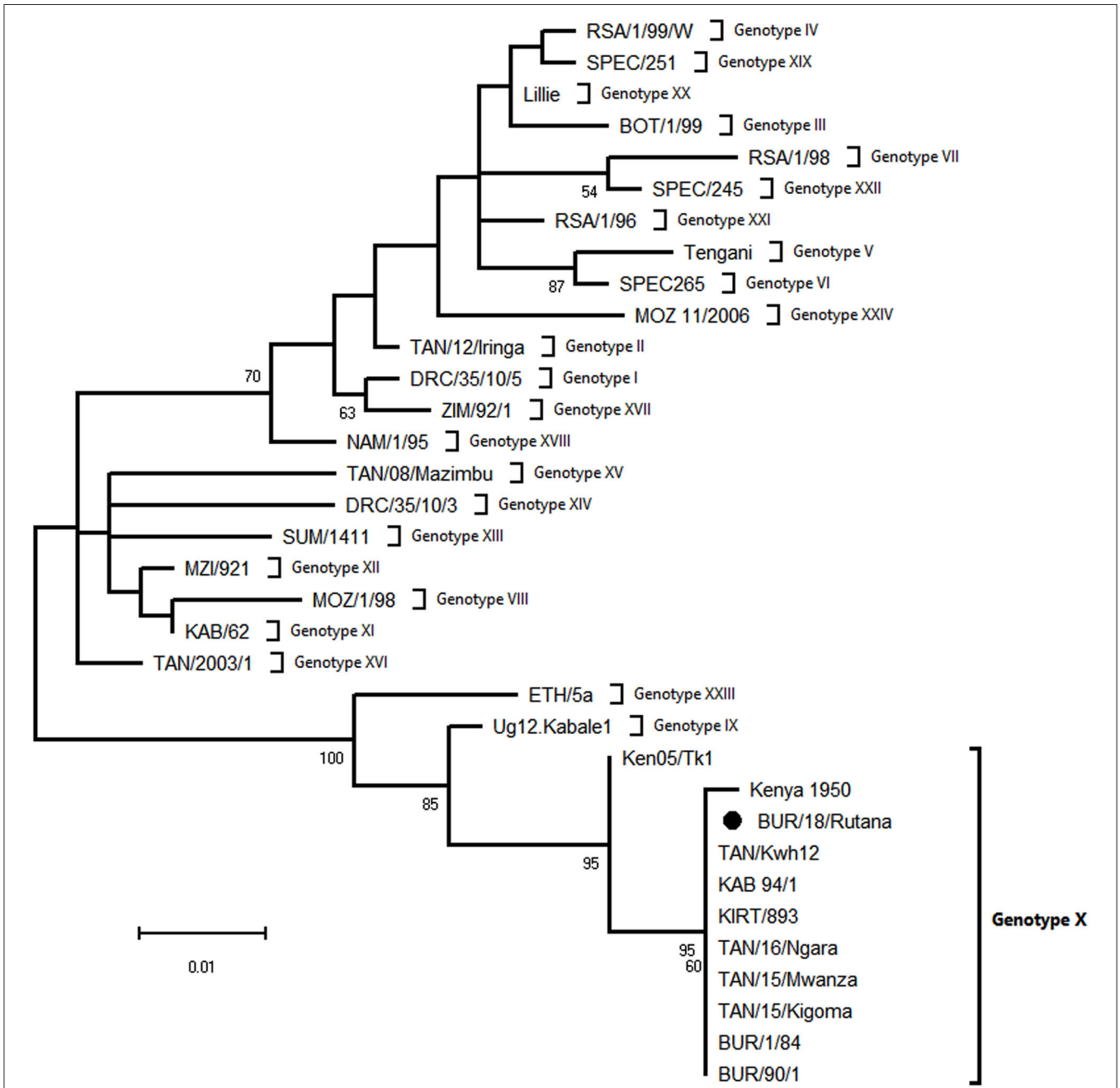

FIGURE 2 | Evolutionary relationships of representative strains of African swine fever virus (ASFV) based on the maximum likelihood phylogeny of the partial p72 gene nucleotide sequences. The phylogenetic analysis was performed using MEGA X (http://www.megasoftware.net) and the Kimura two-parameter substitution model, as determined by a model selection analysis. Phylogeny was inferred following 1,000 bootstrap replications, and the node values show percentage bootstrap support (only values above 50% are shown). The round black spot indicates the ASFV nucleotide sequence from Burundi obtained in this study. The scale bar indicates nucleotide substitutions per site.

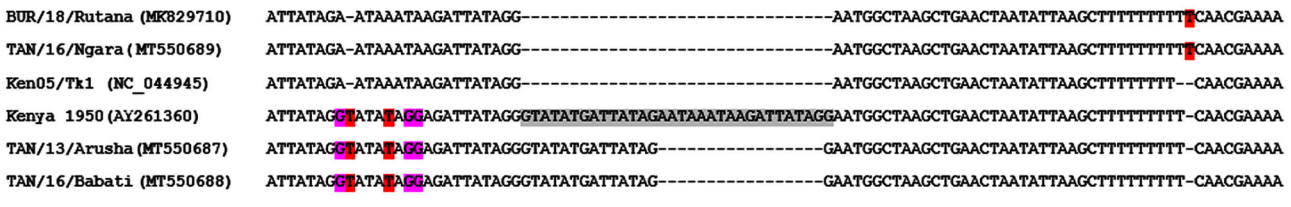

FIGURE 3 | Partial nucleotide sequence alignment of the intergenic region between *I73R* and *I329L* genes in ASFV isolates belonging to *B646L* (p72) genotype X from Eastern Africa. The nucleotides highlighted in gray, present in the reference ASFV strain, are absent in the 2016 Tanzanian ASFV, the 2018 Burundian ASFV, and the tick strain described in Kenya in 2005. Other nucleotide variations between ASFV strains are highlighted in red and pink. The GenBank accession numbers of the nucleotide sequences are shown in parentheses.

as previously described (21, 23, 29). The evolutionary history of ASFV was inferred by the maximum likelihood method using the Kimura two-parameter model implemented in MEGA X (40). Phylogeny was inferred following 1,000 bootstrap replications.

RESULTS

Outbreak Description

The outbreak described in this study was reported in Mutwana village (Giharo district) in August 2018 (**Figure 1**). Afterward, ASF spread from Mutwana to neighboring villages of the Muzye, Butezi, Giharo, and Gakungu zones in the Giharo district before it was reported in villages of the Kinzanza and Gitanga zones of the Gitanga district in September 2018 (**Figure 1**). A total of 3,509 domestic pigs from 1,958 households died in both districts of the Rutana region, South-Eastern Burundi. The main clinical signs presented by affected domestic pigs included anorexia, dyspnea, and congestion of the skin particularly on the peripheral part of the pinna, belly, neck region, and mammary glands, followed by sudden death. Postmortem findings included hydrothorax, splenomegaly, and hemorrhages in the lung, liver, and lymph nodes, especially the hepatogastric and mesenteric lymph nodes.

Confirmation of ASF Using PCR

Each of the lung, liver, and spleen obtained from pigs that naturally died from the disease were tested for the presence of ASFV as previously described (26). All lung, liver, and spleen tissues from the three sampled domestic pigs were found to be positive for ASFV. The spleen had a high ASFV DNA concentration on a nanodrop spectrophotometer, followed by the lung and liver, at 501, 336.5, and 141.5 ng/μL, respectively.

Molecular Characterization of ASFV

The ASFV strain from the Rutana region (South-Eastern Burundi) obtained in this study was designated as BUR/18/Rutana. BLASTn of BUR/18/Rutana *B646L* (p72) ASFV nucleotide sequences in GenBank showed high nucleotide identity to *B646L* (p72) genotype X ASFV strains previously described in Tanzania and Kenya. In order to determine the genetic relationship of BUR/18/Rutana with other ASFVs representing the 24 *B646L* (p72) ASFV genotypes, a phylogenetic tree was constructed with the maximum likelihood method using partial *B646L* (p72) nucleotide sequences. The BUR/18/Rutana ASFV strains clustered together with genotype X strains previously described in Burundi, Tanzania, and Kenya (**Figure 2**).

We amplified the region located between the *I73R* and *I329L* genes, characterized by the presence of TRS. The most similar TRS was that of TAN/16/Ngara responsible for the 2016 ASF outbreak in domestic pigs in the Ngara district of Kagera Region, South-Western Tanzania, followed by that of Ken05/Tk1 collected from a tick in Kenya in 2005 (**Figure 3**). We compared the Kenya 1950 isolate, which is a reference for genotype X, with BUR/18/Rutana. The ASFV strain BUR/18/Rutana lacked a 32 bp fragment in the TRS (**Figure 3**), as was the case for the TAN/16/Ngara and Ken05/Tk1 strains (34). In addition, the Burundian ASFV strain described in this study had the signature AAABNAABA at the *B602L* (CVR) gene and showed 100% amino acid sequence identity to TAN/16/Ngara (**Table 2**).

DISCUSSION

ASF is endemic in Burundi, and 24,696 ASF cases have been reported in the country between January 2005 and December 2018 (13); the disease continues to economically impact on small-scale farmers. In this study, we report an outbreak of a highly fatal hemorrhagic disease of domestic pigs that occurred in 2018 in the Rutana region of Burundi. The presence of ASFV in domestic pigs was confirmed by nucleotide amplification, sequencing, and phylogenetic reconstruction of the ASFV *B646L* (p72) gene, the region located between the *I73R* and *I329L* genes characterized by the presence of TRS, and the *B602L* (CVR) gene. Partial sequencing of the *B646L* (p72) gene is used in order to determine the ASFV genotype. However, to achieve more resolution among closely related strains, analysis of additional ASFV genomic regions is needed (21, 28). Regions with tandem repeat arrays within the coding or in intergenic regions identified in the ASFV genome have proven useful for discerning between closely related ASFV strains (21). Among these regions, the TRS located in the CVR within the *B602L* gene and the TRS located in the intergenic region between the *I73R* and *I329L* genes have been described as suitable to distinguish between closely related ASFV strains and to trace the source of ASF outbreaks (21, 28, 41, 42). Thus, in the present study, *B646L* (p72), *B602L*, and the TRS between the *I73R* and *I329L* genes were analyzed to achieve higher resolution. The results obtained from the present study confirm an ASF outbreak in the Rutana region in South-Eastern Burundi.

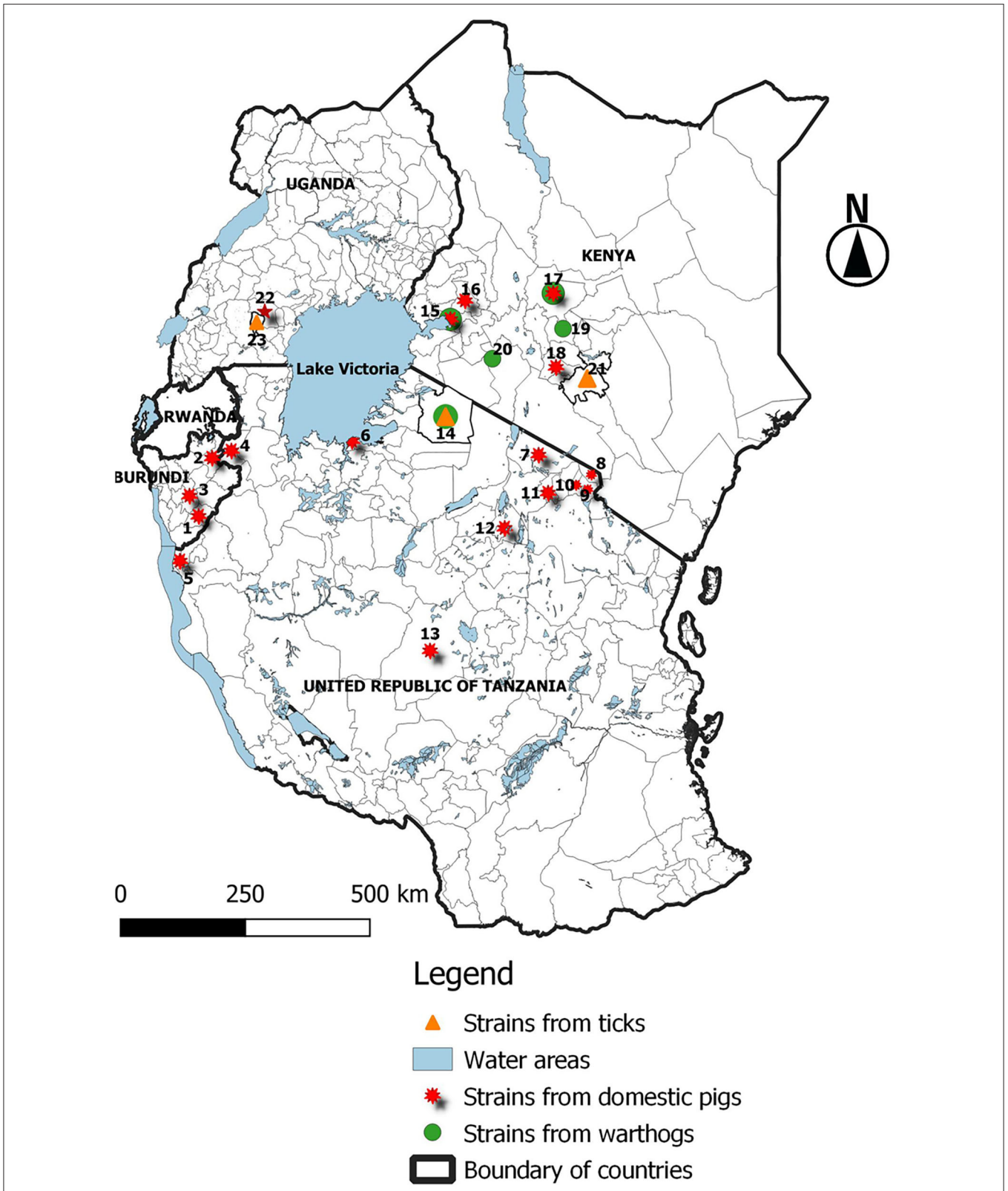

FIGURE 4 | Distribution of ASFV p72 genotype X isolates in the East African Community (EAC) between 1954 and 2018. [1]Rutana 2018 [domestic pig (Dp)]; [2]Muyinga 1990 (Dp); [3]Gitega 1984 (Dp); [4]Ngara 2016 (Dp); [5]Kigoma 2004 and 2015 (Dp); [6]Mwanza 2015 (Dp); [7]Longido 2009 (Dp); [8]Rombo 2013 (Dp); [9]Moshi 2013 (Dp); [10]Machame 2013 (Dp); [11]Arusha 2013 (Dp); [12]Babati 2016 (Dp); [13]Manyoni 2015 (Dp); [14]Kirawira 1968 [warthog (Wh)] and 1989 [Wh and tick (Tk)]; [15]Nyanza 2008 *(Continued)*

FIGURE 4 | (Dp and Wh); [16]Nandi 2005 (Dp); [17]Nanyuki 1954 (Dp), 1959 (Dp and Wh), and 1961 (Dp); [18]Kiambu 2005 (Dp); [19]Kiganjo and Mweiga 1957 and 1959 (Wh); [20]Rift valley 1959 (Wh); [21]Machakos 2005 and 2009 (Tk); [22]SSembabule 1995 (Dp); [23]Lake Mburu national park 2010 (Tk). The isolates from Kenya in 1950 (Kenya 1950) and Uganda in 1964 (Ug64) are not indicated on the map, because their locations are not mentioned in the available literature.

The ASFV responsible for the 2018 outbreak in the Rutana region belonged to *B646L* (p72) genotype X and was closely related to other genotype X strains previously characterized in Burundi, Tanzania, and Kenya (28, 34, 41). Genotype X is one of the predominant ASFV p72 genotypes in countries of the East African Community (**Figure 4**), and it has been isolated from domestic pigs, warthogs, and *Ornothodoros* ticks in the region (23, 39). The ASFV p72 genotype X has been involved in previous outbreaks in Burundi in 1984 and 1990, in Gitega and Muyinga, respectively (5, 21). The ASFV BUR/18/Rutana lacked a 32 bp fragment within TRS compared to the reference genotype X isolate, Kenya 1950 (40). Similarly, the same 32 bp fragment was absent in the ASFV TAN/16/Ngara strain responsible for the ASF outbreak in domestic pigs in South-Western Tanzania in 2016 and the ASFV Ken05/Tk1 strain recovered from a tick that was extracted from a warthog burrow in central Kenya in 2005 (40). In addition, the amino acid tetramer repeats within the CVR of the virus that caused the 2018 ASF outbreak in the Rutana region had the signature AAABNAABA and showed 100% similarity to the virus recovered from the outbreak in Ngara, South-Western Tanzania, in 2016. The amino acid identity was greater with TAN/16/Ngara than with the ASFV strains responsible for earlier outbreaks in Burundi in 1984 and 1990 (21).

The high genetic similarity of the virus described in this study to ASFV strains recovered from domestic pigs, warthogs, and *Ornithodoros* soft ticks vectors is in agreement with previous studies that classified the ASFV p72 genotype X as a sylvatic cycle associated genotype (23, 39, 43). In Burundi, the Ruvubu and Kibira National Parks host warthogs (*Phacochoerus aethiopicus*) and bushpigs (*Potamochoerus porcus*) (18), which are natural reservoirs of ASFV, but the role of the sylvatic cycle in the maintenance and transmission of ASF in the country has not been investigated. Therefore, there is a lack of information on the potential existence of the ASF sylvatic cycle in Burundi, and this aspect should be investigated in wildlife protected areas of Burundi in order to understand the possibility of the virus spilling over from the sylvatic to the domestic cycle. The strain described in this study showed high genetic similarities with ASFV strains previously reported in Burundi and those circulating in the region, indicating regional distribution and circulation of this ASFV genotype. These findings are in agreement with previous studies in the region that have also reported transboundary distribution of different ASFV genotypes including genotype X between the Democratic Republic of the Congo (DRC) and Burundi (44) and genotype II between Malawi, Tanzania, and Zambia (45–48). In these studies, uncontrolled movements of domestic pigs and pork products have been cited as a major factor contributing to the transboundary spread of ASFV strains. Sequence analysis of the three ASFV genomic regions considered in this study showed that the most closely related strain was that responsible for the 2016 ASF outbreak in the Ngara district of Kagera region, South-Western Tanzania, indicating that the same viruses are causing outbreaks on both sides of the Burundi-Tanzania border. Kagera region on the Tanzanian side and the Rutana region in Burundi share borders, and uncontrolled animal movement, including that of domestic pigs, are more likely to happen between these two regions. For instance, movement of refugees together with their livestock, reported in the area (49), can contribute to the spread of animal diseases, including ASF. It has been reported that in order to reduce the economic loss due to ASF outbreaks, some farmers sell their pigs before they show clinical signs as soon as ASF is suspected. This emergency pig sell contributes to the spread of the virus in resource-poor settings, including between countries (50–52). However, considering the proximity of the Rutana region to Ruvubu National Park, where warthogs are present (20), and the reported uncontrolled movement of wild animal species between Ruvubu National Park in Burundi, Akagera National Park in Rwanda, and the Kimisi and Burigi game reserves in Tanzania (49), the virus spillover from the sylvatic to the domestic cycle cannot be excluded based on the results of this study.

This study confirms that the 2018 ASF outbreak in the Rutana region, South-Eastern Burundi, was caused by the ASFV p72 genotype X. The virus showed high genetic similarities with ASFV strains previously described in domestic pigs, warthogs, and soft ticks in East African countries, indicating a possible common wild source and continuous circulation in domestic pigs in the region. This study contributes to the understanding of ASFV epidemiology in Burundi and in the East African Community. It will be interesting to investigate the role of the ASFV sylvatic cycle in Burundi and to perform whole genome sequencing of the ASFV strains reported in this study along with those previously described in Burundi and ASFV strains from neighboring countries to facilitate a better understanding of ASFV dynamics and epidemiology in Eastern and Southern Africa. Such perspective on the changing dynamics may provide an understanding of the global epidemiology of ASF.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

AUTHOR CONTRIBUTIONS

JH, GM, and JN designed the study. JH and LN participated in sample collection and laboratory analysis. JH, GM, and CY analyzed and interpreted data. JH wrote the first draft of the manuscript. JN, LN, CY, OK, DN, GM, and HN reviewed and edited the manuscript. All authors read and approved the

final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper Four

Complete genome analysis of African swine fever virus responsible for outbreaks in domestic pigs in 2018 in Burundi and 2019 in Malawi.

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REGULAR ARTICLES

Complete genome analysis of African swine fever virus responsible for outbreaks in domestic pigs in 2018 in Burundi and 2019 in Malawi

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Abstract
Several African swine fever (ASF) outbreaks in domestic pigs have been reported in Burundi and Malawi and whole-genome sequences of circulating outbreak viruses in these countries are limited. In the present study, complete genome sequences of ASF viruses (ASFV) that caused the 2018 outbreak in Burundi (BUR/18/Rutana) and the 2019 outbreak in Malawi (MAL/19/Karonga) were produced using Illumina next-generation sequencing (NGS) platform and compared with other previously described ASFV complete genomes. The complete nucleotide sequences of BUR/18/Rutana and MAL/19/Karonga were 176,564 and 183,325 base pairs long with GC content of 38.62 and 38.48%, respectively. The MAL/19/Karonga virus had a total of 186 open reading frames (ORFs) while the BUR/18/Rutana strain had 151 ORFs. After comparative genomic analysis, the MAL/19/Karonga virus showed greater than 99% nucleotide identity with other complete nucleotides sequences of p72 genotype II viruses previously described in Tanzania, Europe and Asia including the Georgia 2007/1 isolate. The Burundian ASFV BUR/18/Rutana exhibited 98.95 to 99.34% nucleotide identity with genotype X ASFV previously described in Kenya and in Democratic Republic of the Congo (DRC). The serotyping results classified the BUR/18/Rutana and MAL/19/Karonga ASFV strains in serogroups 7 and 8, respectively. The results of this study provide insight into the genetic structure and antigenic diversity of ASFV strains circulating in Burundi and Malawi. This is important in order to understand the transmission dynamics and genetic evolution of ASFV in eastern Africa, with an ultimate goal of designing an efficient risk management strategy against ASF transboundary spread.

Keywords African swine fever virus · *Asfarviridae* · Burundi · Domestic pig · Malawi · Whole-genome sequencing

Introduction

The aetiology of Africa swine fever (ASF) is ASF virus (ASFV), a linear double-stranded DNA arbovirus with a genome size ranging between 170 and 194 kilobase pairs (kbp), and the only member of the genus *Asfivirus*, family *Asfarviridae* (Alonso et al. 2018). However, a potential new member of the *Asfarviridae* family designated as Abalone asfa-like virus (AbALV) has been recently reported (Matsuyama et al. 2020). The outcome of ASF infection

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in domestic pigs and Eurasian wild boars depends on virulence of causative ASFV and ranges from acute to chronic disease with mortality rates approaching 100% in naïve population (Karger et al. 2019; Pikalo et al. 2019). Due to its high mortality rate, unavailability of a commercial vaccine or effective treatment, and trade restriction of domestic pigs and pork products across countries, ASF is considered as the most serious threat to the global domestic pig industry (Costard et al. 2009; Couacy-Hymann 2019; Onzere et al. 2018). Transmission of ASF is through direct contact between infected and susceptible domestic pigs or wild boars, ingestion of contaminated pork products, contact with infected fomites, indirect transmission through carcasses in the habitat in the case of wild boars, or bites by infected soft ticks of the *Ornithodoros moubata* complex (Chenais et al. 2018; Penrith and Vosloo 2009). Soft ticks of the *O. moubata* complex act as vectors of the ASFV while in eastern and southern Africa, asymptomatically infected wild suids mainly warthogs (*Phacochoerus africanus*) play an important role as ASFV reservoirs (Jori et al. 2013). The ASFV infection of other wild suids species such as bush pig (*Potamochoerus larvatus*) and giant forest hogs (*Hylochoerus meinertzhageni*) has been previously reported but their role in the epidemiology of the virus is not well known (Penrith et al. 2019).

Domestic pigs and the pig farming systems in Africa, South of the Sahara, have been reported to play an important role in the ASFV transmission and spread (Mwiine et al. 2019; Yona et al. 2020) while the high stability of ASFV in pork products is cited to be the major factor of ASFV spread across long distances. For instance, the first escape of the virus from Africa to Portugal in 1957 and again in 1960 was associated to airplane waste with contaminated pork products that was used for pig feeding while contaminated ship waste was cited to be the origin of ASFV introduction in Georgia in 2007 (Rowlands et al. 2008). More than 33 countries of Africa, South of the Sahara, have reported ASF where the disease is endemic and ASFV is becoming more prevalent in European and Asian countries threatening global food and nutritional security (Ge et al. 2019; Penrith et al. 2019).

The ASFV genome varies in size between 170 and 194 kilobase pairs (kbp) with a conserved central region of about 125 kbp, in addition to the left variable region (LVR) of 38 to 47 kbp and the right variable region (RVR) of 13 to16 kbp (de Villiers et al. 2010). The variation of the genome lengths of different ASFV strains is caused by the gain or loss of members of the five different multigene families (MGF) of ASFV found in the LVR and the RVR, for instance, MGFs 100, 110, 300, 360 and 530/505 (Alonso et al. 2018). Previous studies have reported between 151 and 167 ORFs in ASFV genomes (de Villiers et al. 2010). However, an increasing number of studies have reported more than 167 ORFs in ASFV genomes especially the strains belonging to ASFV p72 genotype II, including seven Polish isolates, collected between 2016 and 2017 with 187 to 190 ORFs (Mazur-Panasiuk et al. 2019) and the ASFV strain Belgium/Etalle/wb/2018 detected in wild boar in Belgium in 2018 with 186 ORFs (Gilliaux et al. 2019). A study that analyzed 12 complete genomes of the ASFV strains collected in Sardinia, Italy, from 1978 to 2014 reported 231 ORFs in four isolates and 235 ORFs in eight ASFV isolates with 66 ORFs defined as uncharacterized (Torresi et al. 2020).

Based on partial nucleotide sequence analysis of the *B646L* gene that encodes for the major capsid protein p72, 24 (I–XXIV) ASFV genotypes have been identified and all of these have been reported to circulate in Africa, South of the Sahara (Achenbach et al. 2017; Lubisi et al. 2007; Quembo et al. 2018). Previous studies have reported ASFV p72 genotypes II, V, VIII and XII in Malawi while only ASFV p72 genotype X was reported in Burundi (Hakizimana et al. 2020a, b; Lubisi et al. 2005). Currently, only 3 complete and fully annotated ASFV strains belonging to p72 genotype X are available in the GenBank, including two strains from Kenya and one from Democratic Republic of the Congo (DRC) (Bisimwa et al. 2021; de Villiers et al. 2010). However, despite the endemic status of ASF in Burundi, no ASFV has been fully sequenced. In addition, there is no ASFV p72 genotype II strain from Malawi that has been subjected to complete genome sequencing. In this study, we report the complete genome sequences of ASFV p72 genotype X (BUR/18/Rutana) responsible for the 2018 ASF outbreak in Burundi and ASFV 72 genotype II (MAL/19/Karonga) that caused an outbreak during 2019 in Malawi.

Materials and methods

Sequencing of the ASFV complete genome

Collection of the samples used in this study and subsequent ASF confirmation and genotyping have been previously described (Hakizimana et al. 2020a, b). Viral DNA was extracted from tissue samples using the Quick-DNA™ Miniprep Plus Kit (Zymo Research Corporation, CA, USA), following the manufacturer's instructions. Assessment of the integrity and quality of the extracted DNA was done through 1% agarose gel electrophoresis for 30 min running at 160 V with 0.5µL of sample DNA loaded. The starting genomic DNA for complete genome sequencing was quantified by picogreen method (Invitrogen, Catalog # P7589) using Victor 3 fluorometry (PerkinElmer Life and Analytical Sciences, Shelton, USA). Illumina NovaSeq6000 instrument with 2×150 bp configuration was used for sequencing and TruSeq Nano DNA Kit (Catalog # 20,015,964) was used for

library preparation, according to the manufacturer's protocol. Quality control of the prepared library was done by 2100 Bioanalyzer using a DNA 1000 chip (Agilent Technologies, USA) while the library quantification was performed using real-time polymerase chain reaction (qPCR) according to the Illumina qPCR Quantification Protocol Guide (Catalog # SY-930–1010). The libraries were subjected to sequencing to produce approximately 28 million paired-end reads (4 GB) per sample.

Assembly and annotation of the ASFV genome

Adapter sequences and low-quality reads trimming were performed using Trim Galore version 0.6.4 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with cutadapt version 2.8 and the quality Phred score cutoff was set to 30 with a minimum reads length of 75 nucleotides. The quality of the filtered sequence data was assessed using FastQC version 0.11.9 (Andrews 2010). The quality-filtered reads were de novo assembled using SPAdes version 3.13.1 (Bankevich et al. 2012) and Megahit version 1.2.9 (Li et al. 2015). The assembly contigs were mapped to the reference genome using Burrows-Wheeler Aligner (BWA) version 0.7.17 with maximum exact match (mem) option (Li 2013) and the QUAST program version 5.0.2 (Gurevich et al. 2013) was used to evaluate the quality of the assembly. The longest overlapping scaffolds were assembled to generate the ASFV complete genomes. The Genome Annotation Transfer Utility (GATU) software (Tcherepanov et al. 2006) was used for annotation of the assembled ASFV genomes using Georgia 2007/1 (GenBank accession number NC_044959.2) and Ken05/Tk1 (GenBank accession number NC_044945.1) as reference genomes. The basic local alignment search tool for nucleotide (BLASTN) version 2.11.0 + (Zhang et al. 2000) was used for pairwise nucleotide alignment and search for nucleotide identity at GenBank nucleotide database. Multiple sequence alignment was carried out using MAFFT program version 7.221 (Katoh and Standley 2013) and the evolutionary history was inferred using the maximum likelihood method with 1000 bootstrap replications and evolutionary distances were calculated using Kimura 2-parameter model (Kimura 1980) as implemented in MEGA X (Kumar et al. 2018).

Results

Characteristics of the complete genomes of Burundian and Malawian ASFV strains

Complete genome sequences of the ASFV strains responsible for the 2018 outbreak in Rutana region, South-eastern Burundi (BUR/18/Rutana), and the 2019 outbreak in Karonga district, northern Malawi (MAL/19/Karonga), were determined in this study. The strains BUR/18/Rutana and MAL/19/Karonga belong to ASFV p72 genotypes X and II, respectively, as previously described through partial genome amplification and sequencing targeting specific genomic regions (Hakizimana et al. 2020a, b). The complete genome assembly generated genomes of 176,564 bp for BUR/18/Rutana and 183,325 bp for MAL/19/Karonga with GC content of 38.62 and 38.48%, respectively. The MAL/19/Karonga strain had a total of 186 open reading frames (ORFs) while the BUR/18/Rutana strain had 151 ORFs as highlighted by the whole-genome alignment of homologous genes between the ASFV strains described in this study and the corresponding reference genomes (Fig. 1). For MAL/19/Karonga, a total of 44 multigene family (MGF) members were identified within the genome including MGF 100 (3 members), MGF 110 (10 members), MGF 300 (3 members), MGF 360 (18 members) and MGF 505 (10 members). Furthermore, 36 MGF members were identified within the genome of BUR/18/Rutana strain including MGF 100 (1 member), MGF 110 (8 members), MGF 300 (3

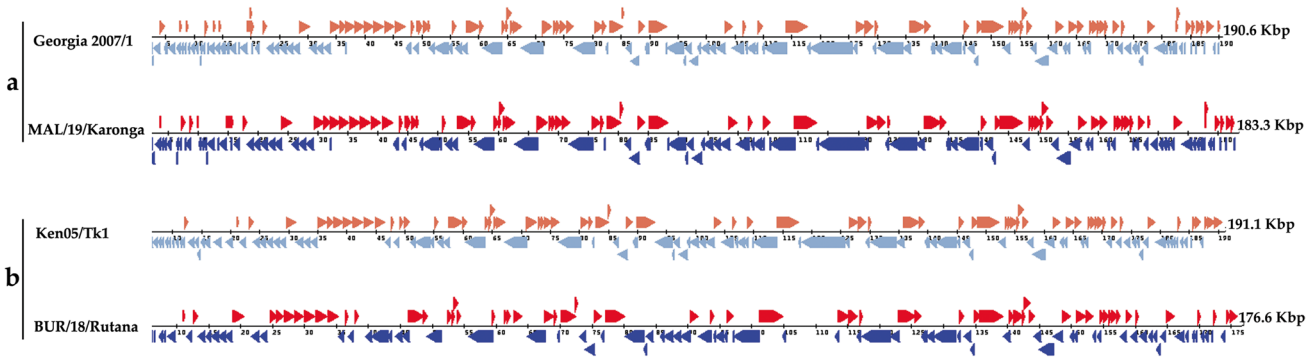

Fig. 1 Graphical display of African swine fever virus open reading frames (ORFs) of MAL/19/Karonga (**a**) and BUR/18/Rutana (**b**.) automatically annotated by Genome Annotation Transfer Utility (GATU) using corresponding very closely related African swine fever virus reference genomes. The direction of arrows indicates the 5′ to 3′orientation of ORFs

members), MGF 360 (16 members) and MGF 505 (8 members). The complete genome sequences generated in this study were submitted to GenBank and assigned accession numbers (MW856067 for BUR/18/Rutana and MW856068 for MAL/19/Karonga).

Comparative genomic analysis

Using complete genome sequences for BLASTN search at the GenBank, the MAL/19/Karonga virus was closely related to Tanzania/Rukwa/2017/1 (GenBank accession number LR813622) ASFV strain collected in South-western Tanzania from an infected domestic pig during an ASF outbreak in 2017 and belonging to ASFV p72 genotype II, with 99.97% nucleotide identity. The percentage of nucleotide identity was greater than 99% with other complete genomic sequences of ASFV belonging to p72 genotype II isolated in Europe and Asia including the Georgia 2007/1 isolate. On the other hand, the BUR/18/Rutana ASFV strain exhibited 99.34%, 99.08% and 98.95% nucleotide identity with the Uvira B53 (Bisimwa et al. 2021), Ken05/Tk1 (Bishop et al. 2015) and Kenya 1950 (GenBank accession number AY261360) ASFV p72 genotype X strains, respectively (Table 1). Phylogenetic reconstruction using complete genomes clustered the MAL/19/Karonga and BUR/18/Rutana viruses into ASFV genotypes II and X, respectively (Fig. 2). With a genome size of 183,325 bp, the MAL/19/Karonga strain was 139 bp longer than the Tanzania/Rukwa/2017/1 (183,186 bp) and about 6 to 7 kbp shorter than some ASFV p72 genotype II isolates available in the GenBank nucleotide database, for instance Georgia 2007/1 (190,584 bp), Arm/07/CBM/c2 (190,145 bp) and ASFV-wbBS01 (189,394 bp). Furthermore, the BUR/18/Rutana strain with the genome length of 176,564 bp was about 4 to 17 kbp shorter than the Uvira B53 (180,916 bp), Ken05/Tk1 (191,058 bp) and Kenya 1950 (193,886 bp) ASFV p72 genotype X genomes. The difference in genome length is due to differences within some genes and MGF members. For instance, among the 186 ORFs identified in MAL/19/Karonga, 151 ORFs had 100% nucleotide identity with their homologues in the reference genome while the remaining 35 ORFs were polymorphic with nucleotide identity with the reference genome varying from 60 to 99.9%. In addition, four ORFs (MGF 360-1Lb, ASFV G ACD 00,120, MGF 110-7L and MGF 110–10-L—MGF110-14L fusion) present in ASFV reference genome were below the 60% nucleotide identity threshold and considered absent in the MAL/19/Karonga virus. Among the 151 ORFs identified in BUR/18/Rutana virus, only 35 ORFs had 100% nucleotide identity with their homologues in Ken05/Tk1 reference genome while the remaining ones had between 60 and 99.9% identity. Besides, 10 MGF members (MGF 110-7L, MGF 110-8L, MGF 100-1R, MGF 110-9L, MGF 110-11L (FRAG-2), MGF 110-13L-14L, MGF 360-12L, MGF 360-15R, MGF 100-3L, MGF 360-18R) present in the Ken05/Tk1 reference genome had a nucleotide similarity below 60% compared to their homologues in BUR/18/Rutana virus.

Determination of the serogroups of Burundian and Malawian ASFV strains based on EP402R (CD2v) gene sequences

In order to classify the ASFV strains described in this study among the eight previously determined serogroups based on the ASFV hemadsorption inhibition (HAI) properties, we compared sequences of the *EP402R* gene that encodes the CD2v major ASFV antigen protein between them and selected isolates representing each serogroup retrieved from GenBank. A high nucleotide sequence variation was observed among the compared sequences and the serotyping results classified the BUR/18/Rutana and MAL/19/Karonga ASFV viruses in serogroups 7 and 8, respectively (Fig. 3). The Burundian ASFV strain grouped together with two strains belonging to serogroup 7 previously described, for instance the Uvira B53 ASFV strain collected during an ASF outbreak in South Kivu province of the Democratic Republic of the Congo (DRC) in 2019 and the Uganda ASFV strain (Bisimwa et al. 2021; Malogolovkin et al. 2015a, b), whereas the MAL/19/Karonga ASFV strain clustered together with strains belonging to ASFV serogroup 8 previously described in Europe and Asia.

Discussion

The limited knowledge of the genetic variation of the ASFV has hindered the development of effective control and prevention strategies, including vaccine, diagnostic test and antiviral treatment development (Arabyan et al. 2019; Bao et al. 2021; Torresi et al. 2020; Urbano et al. 2021). Partial nucleotide sequencing of specific ASFV genomic regions is conventionally used to determine ASFV genotypes and to discriminate closely related ASFV strains. However, in order to obtain adequate information on transmission dynamics, genetic variation and molecular evolution of different ASFV strains, complete genome sequencing is required. To date, despite the regular reports of the ASFV p72 genotype II in different countries of eastern and southern Africa, only one fully annotated complete genome of the genotype II from those countries is publicly available, for instance the Tanzania/Rukwa/2017/1 collected in South-western Tanzania from an infected domestic pig during an outbreak in 2017 (Njau et al. 2021). There is no ASFV p72 genotype II strain from Malawi that has been subjected to complete genome sequencing and no ASFV strain from Burundi that has been fully sequenced. In the present study, complete genome

Table 1 Publicly available complete genome sequences of African swine fever virus strains from Africa and selected strains from Europe and Asia used for comparative genomic analysis in this study

Name of the strain	GenBank accession number	Country of origin	Year of collection	p72 genotype	Length (bp)	Percentage of identity with MAL/19/Karonga (%)	Percentage of identity with BUR/18/Rutana (%)	Host species	Reference
Mkuzi1979	AY261362	South Africa	1979	I	192,714	97.95	93.35	Tick	Unpublished
Benin 97/1	NC_044956	Benin	1997	I	182,284	97.91	93.01	Domestic pig	(Chapman et al., 2008)
Liv13/33(OmLF2)	MN913970	Zambia	1983	I	188,277	98.29	93.29	Tick	(Chastagner et al. 2020)
MAL/19/Karonga	MW856068	Malawi	2019	II	183,325	100	93.42	Domestic pig	This study
Arm/07/CBM/c2	LR812933	Armenia	2007	II	190,145	99.95	93.45	Domestic pig	Unpublished
Georgia 2007/1	NC_044959.2	Georgia	2007	II	190,584	99.95	93.45	Domestic pig	(Chapman et al. 2011)
ASFV-wbBS01	MK645909	China	2018	II	189,394	99.90	93.44	Wild boar	Unpublished
Tanzania/Rukwa/2017/1	LR813622	Tanzania	2017	II	183,186	99.97	93.44	Domestic pig	(Njau et al. 2021)
Warmbaths	AY261365	South Africa	1987	III	190,773	96.83	93.45	Tick	Unpublished
Warthog	AY261366	Namibia	1980	IV	186,528	97.50	93.11	Warthog	Unpublished
Tengani62	AY261364	Malawi	1962	V	185,689	96.72	93.34	Domestic pig	Unpublished
MalawiLil-20/1(1983)	AY261361	Malawi	1983	VIII	187,612	94.62	93.42	Tick	Unpublished
Ken06.Bus	NC_044946	Kenya	2006	IX	184,368	92.78	97.71	Domestic pig	(Bishop et al. 2015)
R35	MH025920	Uganda	2015	IX	188,629	92.79	97.71	Domestic pig	(Masembe et al. 2018)
BUR/18/Rutana	MW856067	Burundi	2018	X	176,564	93.42	100	Domestic pig	This study
Ken05/Tk1	NC_044945	Kenya	2005	X	191,058	93.74	99.08	Tick	(Bishop et al. 2015)
ASFV Ken.rie1	LR899131	Kenya	2019	X	189,950	93.49	98.98	Tick	Unpublished
Uvira B53	MT956648	DRC	2019	X	180,916	92.26	99.34	Domestic pig	(Bisimwa et al. 2021)
Kenya 1950	AY261360	Kenya	1950	X	193,886	93.64	98.95	Domestic pig	Unpublished
Zaire	MN630494.2	DRC	1977	XX	184,820	96.68	93.77	Domestic pig	(Ndlovu et al. 2020a)
RSA_2_2004	MN641877.2	South Africa	2004	XX	189,903	95.27	92.22	Wild boar	(Ndlovu et al. 2020a)
RSA_2_2008	MN336500.3	South Africa	2008	XXII	190,066	94.17	90.50	Tick	(Ndlovu et al. 2020b)

DRC, Democratic Republic of the Congo

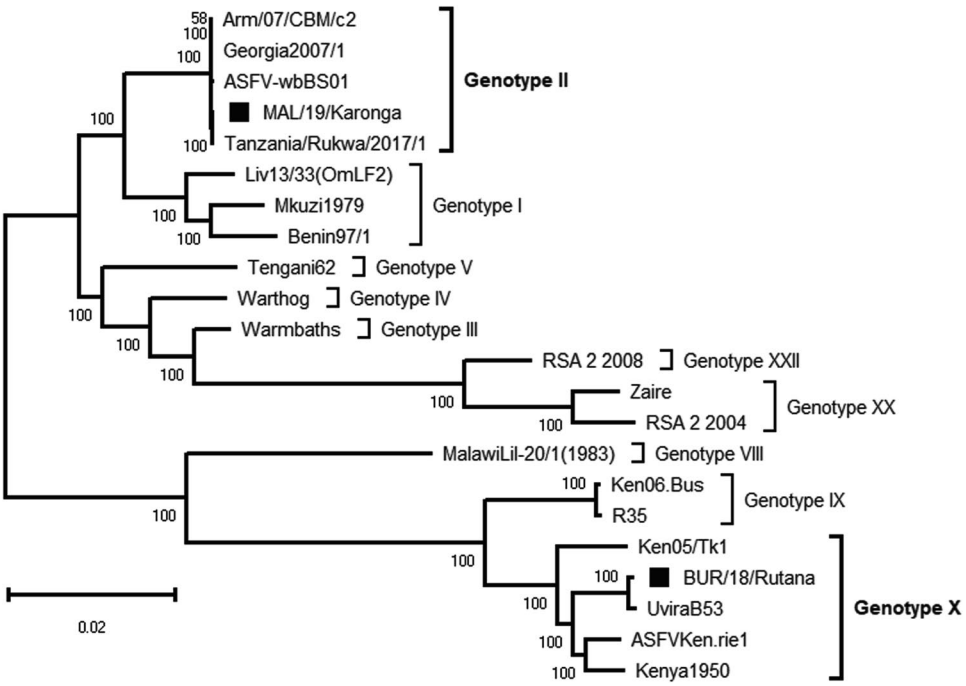

Fig. 2 Maximum likelihood phylogenetic tree obtained after multiple sequence alignment of complete genomes of African swine fever virus strains from Africa and selected strains from Europe and Asia. The viruses described in this study are indicated by black squares and the scale bar indicates nucleotide substitution per site while the node values show percentage of bootstrap support. The analysis involved 22 nucleotide sequences with a total of 166,578 positions in the final dataset

sequences of the ASFV p72 genotype X responsible for the 2018 outbreak in Burundi and genotype II virus that caused the 2019 ASF outbreak in Malawi were generated using Illumina NGS technology. The complete genome sequences generated in this study were closely related to ASFV strains previously described, available in the GenBank database, belonging to ASFV p72 genotype X for the BUR/18/Rutana strain from Burundi and to genotype II for the MAL/19/Karonga strain from Malawi. Besides, serotyping results classified the BUR/18/Rutana and MAL/19/Karonga ASFV strains into ASFV serogroups 7 and 8, respectively.

The Burundian ASFV strain was more closely related to Uvira B53 ASFV strain collected during an ASF outbreak in in South Kivu province of the DRC (Bisimwa et al. 2021), with 99.34% nucleotides identity. These findings are in agreement with the results of studies using partial nucleotide sequencing where relatedness between those two ASFV strains were reported (Bisimwa et al. 2020; Hakizimana et al. 2020b) highlighting the possibility of transboundary spread of genotype X viruses between Burundi and DRC, as previously speculated. Furthermore, the Malawian ASFV strain described in this study was more closely related to the Tanzania/Rukwa/2017/1 ASFV strain collected in South-western Tanzania from an infected domestic pig during an ASF outbreak in 2017 (Njau et al. 2021), with 99.97% nucleotide identity. The high nucleotide similarity between ASFV p72 genotype II strains circulating in Malawi and Tanzania has been previously reported by studies using partial nucleotide sequencing suggesting a common source and transboundary spread of ASFV between these two countries (Hakizimana et al. 2020a; Misinzo et al. 2012). In addition, the Malawian ASFV strain had more than 99% nucleotides identity with ASFV p72 genotype II viruses previously described in Europe and Asia suggesting a possible common ancestor of these ASFV strains as previously speculated (Hakizimana et al. 2020a; Misinzo et al. 2012; Quembo et al. 2018; Rowlands et al. 2008).

Comparative genomic analysis revealed genetic variation in the ASFV strains described in this study compared to

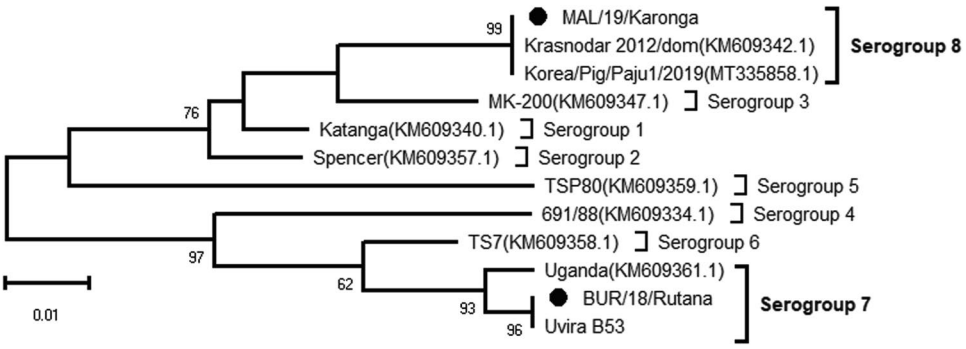

Fig. 3 A maximum likelihood phylogenetic tree of the ASFV *EP402R* (CD2v) gene indicating serogroups of selected ASFV strains. The strains responsible for the 2018 and 2019 ASF outbreak in Burundi and in Malawi are indicated by a black dot. The scale bar indicates the number of substitutions per site

ASFV genomes previously described available in the GenBank. For instance, the *DP96R* gene reported as absent in the Uvira B53 ASFV strain was present in BUR/18/Rutana and MAL/19/Karonga strains with 93.6% and 100% nucleotide identity with the Ken05/Tk1 ASFV p72 genotype X and Georgia 2007/1 ASFV p72 genotype II reference genomes, respectively. The *DP96R* gene encodes the UK protein potentially involved in determining the ASFV virulence in domestic pigs (Zsak et al. 1998) and its presence in BUR/18/Rutana and MAL/19/Karonga ASFV strains may explain the high virulence of these strains as evidenced by high mortality rate during the 2018 and 2019 ASF outbreaks in Rutana region of Burundi and Karonga district in northern Malawi, as previously described (Hakizimana et al. 2020a, b). In addition, the *K196R* and the *B119L (9GL)* genes encoding the thymidine kinase and sulfhydryl oxidase enzymes, respectively, also described as the factors of virulence for ASFV (Rodríguez et al. 2015) were present in BUR/18/Rutana and MAL/19/Karonga ASFV strains.

Previous studies have reported important genetic variation within the members of the MGFs located at the both ends of the ASFV genome resulting in difference of the genome size of different ASFV strains (Torresi et al. 2020; Urbano et al. 2021). In the present study, several single-nucleotide polymorphisms (SNPs) and complete ORF deletion were observed within different MGF members. For instance, four MGF members (MGF 100-1R, MGF 110-7L, MGF 110-8L and MGF 110-9L) absent in the BUR/18/Rutana strains were also missing in the Uvira B53 strains as previously reported (Bisimwa et al. 2021). The MGF 360-1Lb gene was truncated in the MAL/19/Karonga strain and the same observation was reported in China/2018/AnhuiXCGQ ASFV strain collected during an ASF outbreak in domestic pigs in Anhui province of China in September 2018 (Bao et al. 2019). In addition, a deletion of almost all members of the MGF 110 were reported in the Estonia 2014 ASFV strain (Zani et al. 2018). The impact of these genetic variations on the phenotypes of the ASFV strains described in this study is subject to further investigations.

The protein pEP402R, a homologue of the T-lymphocyte surface antigen CD2, encoded by the *EP402R* gene is located in the lipoprotein membrane of the outer viral envelope and plays an important role in the adhesion of erythrocytes to infected cells (hemadsorption) and the binding of the ASFV particles to host erythrocytes during infection (Alejo et al. 2018; Dixon et al. 2019). This gene has been used to define eight viral antigenic types called serogroups (Malogolovkin and Kolbasov 2019). The results of the present study showed that the BUR/18/Rutana and MAL/19/Karonga ASFV strains may share the hemadsorption properties with ASFV strains belonging to serogroups 7 and 8, respectively (Fig. 3). It has been reported that the ASFV isolates classified into the same serotype show cross-protection responses from challenge during the vaccine development experiments (Malogolovkin et al. 2015a, b; Sánchez et al. 2019). Thus, the determination of the ASFV serogroups was suggested as a perfect tool for discriminating ASFV strains with different virulence and prediction of the efficacy of a specific ASFV vaccine (Burmakina et al. 2016). Recently, genetic signatures specific to each ASFV serotype have been described with the potential of elucidating more on the genetic and antigenic diversity of the ASFV (Malogolovkin et al. 2020; Urbano et al. 2021). Interestingly, the ASFV strains described in this study had the PPPKPC amino acid sequences repeated 4 and 3 times in the BUR/18/Rutana and MAL/19/Karonga ASFV strains, respectively. Similar tandem amino acid repeat sequences within the *EP402R* (CD2v) gene were reported in the Uvira B53 ASFV strain (Bisimwa et al. 2021).

In conclusion, the results of this study provided important insight into the genetic structure of the ASFV p72 genotype X responsible for the 2018 outbreak in Burundi and genotype II virus that caused the 2019 ASF outbreak in Malawi. Additionally, the strains BUR/18/Rutana and MAL/19/Karonga were classified into ASFV serogroups 7 and 8, respectively. These results will serve as backbone for possible future investigations concerning molecular evolution, transmission dynamics, diagnostic improvement and control strategies for ASFV.

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Availability of data and material The nucleotide sequences generated in the present study were submitted to the NCBI GenBank with accession numbers MW856067 and MW856068.

Declarations

The samples used in this study were collected from naturally dead domestic pigs according to common veterinary practice as part of routine veterinary investigation in Burundi and Malawi. In Burundi, sample collection was done according to the Burundian Animal Health Law (Law No. 1/28 of 24th December 2009) and the Law No. 1/06 of 21st

March 2011 related to the practice of veterinary medicine in Burundi while in Malawi, the Control and Diseases of Animals Act (CAP 66:02 of 1967) and the rule 6 of the Swine Fever Rules G.N. 209/1968 were followed. Oral consent was obtained from the domestic pig owners before sampling of their dead domestic pigs and documented in the Veterinary Officer registry.

Conflict of interest The authors declare no competing interests.

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CHAPTER THREE

General Discussion, Conclusion and Recommendations

3.1 Discussion

The complex epidemiology of ASF in eastern and southern Africa where all 24 described ASFV genotypes exist, necessitates the use of molecular epidemiology to support control and prevention strategies. The ASF situation in Burundi and Malawi is poorly documented with 273 ASF outbreaks affecting 103 015 domestic pigs that led to 90 833 deaths in both countries, as reported to the World Organization for Animal Health (OIE) between January 2005 and December 2019 (OIE, 2020). Despite frequent ASF outbreaks in domestic pigs in Burundi and Malawi, genetic characteristics of ASFV in these countries is limited. For instance, most of ASFV strains genetically characterized in Burundi and Malawi were collected more than two decades ago (Bastos *et al*., 2003; Lubisi *et al*., 2005; Nix *et al*., 2006). This thesis provides a recent update of the ASF situation in Burundi and Malawi with a special focus on outbreak investigation and molecular characterization of ASFV. In addition, this is the first study to report the complete genome sequences of ASFV from Burundi and that of ASFV genotype II from Malawi. The findings of this study are crucial to inform surveillance and control programs and provide a baseline for future research of ASF in Burundi, Malawi and other countries in eastern and southern Africa.

The first paper discussed in this thesis used phylodynamic approach to investigate the evolutionary history and transmission dynamics of ASFV in selected countries of eastern and southern Africa. A high ASFV genotypic diversity with several transboundary spread events were observed between concerned countries. These findings are in agreement with previous studies that investigated ASFV in eastern and southern Africa (Mulumba-Mfumu *et al*., 2019; Penrith *et al*., 2019). The high genotypic diversity of ASFV in eastern and southern Africa is attributed to the existence of ASFV sylvatic cycle in this area with the ASFV natural reservoirs, for instance wild suids (mainly warthogs) and soft ticks of the *Ornithodoros moubata* complex inhabiting warthogs burrows (Penrith *et al*., 2019). Countries of eastern and southern Africa are rich in wildlife protected area where cohabitation of warthogs and *Ornithodoros moubata* exists and represents hotspots for ASFV transmission from wildlife to domestic pigs (Jori *et al*., 2013; Quembo *et al*., 2018). The role of ASFV sylvatic cycle in ASFV genetic diversity, maintenance and spread has

been previously reported (Quembo *et al*., 2018). Recently, it has been shown that ticks of the *Ornithodoros* species harbour parts of the ASFV genetic elements. These ASFV-like integrated (ASFLI) elements seem to protect ticks against ASFV infection and if a tick contains ASFLI of a certain genotype of ASFV, it becomes immune to being infected by that genotype. Therefore, the ASFLI type present in a particular tick species, determines the distribution of ASFV in a particular location (Forth *et al*., 2020). Among the 15 ASFV genotypes found to circulate between Tanzania and its eight neighbouring countries, 13 were isolated from ASFV sylvatic cycle highlighting the importance of wildlife protected areas in the epidemiology of ASF in eastern and southern Africa. The evolution rate of 4.805×10^{-5} substitution/site/year obtained in this study was closely related to the substitution of rapidly evolving RNA viruses and in agreement with the evolution rate estimated for ASFV elsewhere (Duffy *et al*., 2008; Michaud *et al*., 2013; Alkhamis *et al*., 2018). In addition, the time to the most recent common ancestor (TMRCA) of 1652.233 (1626.473, 1667.735) estimated in this study supported the assumption that ASFV may have been circulating in eastern Africa before its first description in 1921 (Michaud *et al*., 2013; Alkhamis *et al*., 2018).

The second paper investigated the transmission dynamics of ASFV in Malawi using standardized molecular approach targeting the *B646L* (p72) gene, the intergenic region (IGR) between *I73R* and *I329L* genes and the central variable region (CVR) coded by *B602L* gene. The ASFV responsible for the 2019 outbreak in northern Malawi clustered together with ASFV p72 genotype II with high genetic similarity with viruses circulating in neighbouring countries, Europe and Asia. The possibility of transboundary transmission of ASF between Malawi, Tanzania and Zambia was highlighted considering the high genetic similarity of the isolates, the proximity of towns where this ASFV genotype II was isolated and the shared borders between those countries. The introduction of ASFV genotype II into Tanzania in 2010 from Malawi was previously speculated (Misinzo *et al*., 2012) and since then it has been spreading northward in Tanzania devastating the livelihood of domestic pig farmers, food and nutritional security (Fasina *et al*., 2020; Yona *et al*., 2020). There is a need for application of strict control measures to prevent the northward spread of this ASFV genotype II, otherwise, it will reach neighbouring countries such as Burundi, Kenya, Rwanda and Uganda.

The third manuscript describes the identification and characterization of the ASFV circulating in Burundi based on partial amplification and nucleotide sequencing of specific viral genomic regions. After phylogenetic analysis, the ASFV collected in Burundi in 2018 clustered with ASFV p72 genotype X and was closely related to viruses previously described in Tanzania, Kenya and DRC highlighting the distribution of this genotype at regional level. The ASFV genotype X is widely distributed in eastern Africa where it has been isolated from both sylvatic and domestic cycles of ASFV (Gallardo *et al*., 2011; Misinzo *et al*., 2014; Bishop *et al*., 2015). In addition, previously characterized ASFV isolates from the 1984 and 1990 outbreaks in Burundi belonged to ASFV p72 genotype X (Lubisi *et al*., 2005; Nix *et al*., 2006) suggesting persistent circulation of this ASFV genotype in Burundi. Further investigations into the ASFV sylvatic cycle in Burundi are recommended in order to understand the role of the sylvatic cycle in the maintenance of ASFV in the country.

The fourth manuscript discussed in this thesis reports the complete genome sequences of ASFV strains from Burundi and Malawi obtained using Illumina next-generation sequencing platform. The availability of complete genome sequences of ASFV from Africa, south of the Sahara is limited and this study contributes critical information for ASFV related research. The complete genome sequences of ASFV strains BUR/18/Rutana and MAL/19/Karonga collected in 2018 in Burundi and in 2019 in Malawi, respectively were described and compared with other previously described ASFV complete genomes. The additional information on ORFs based on complete genomes and serotyping based on *EP402R* (CD2v) gene sequences make this manuscript interesting. This study demonstrated high genetic similarity between the ASFV responsible for outbreak in Karonga district in 2019 and the isolate collected during an ASF outbreak in 2017 in Tanzania (Njau *et al*., 2021). Besides, high genetic similarity was observed with ASFV complete genomes previously reported in Europe and Asia supporting the assumption of possible common wild source of ASFV genotype II circulating globally (Rowlands *et al*., 2008; Misinzo *et al*., 2012; Quembo *et al*., 2018). Based on the *EP402R* (CD2v) gene sequences, the strains BUR/18/Rutana and MAL/19/Karonga grouped into ASFV serogroups 7 and 8, respectively. The *EP402R* gene is used to classify the ASFV into eight serogroups and cross-protection between isolates of the same serogroup has been reported during the experiments of vaccine development (Malogolovkin *et al*., 2015). Thus, the classification of the ASFV into serogroups is recommended as a suitable tool for

discerning different ASFV isolates and prediction of the effectiveness of a potential vaccine of ASFV (Burmakina *et al*., 2016).

The results of this study suggest persistent circulation of ASFV in concerned countries and provide important insights into the genetic structure and antigenic diversity of ASFV strains circulating in Burundi and Malawi. This is important in order to understand the transmission dynamics and genetic evolution of ASFV in eastern and southern Africa, with an ultimate goal of designing an efficient risk management strategy against ASF transboundary spread. However, further scientific investigation into sylvatic cycle of ASFV in countries of eastern and southern Africa is needed for a comprehensive understanding of the epidemiology of ASFV in the region.

3.2 Conclusion

The design of an efficient risk management strategy against the transboundary spread of ASF has been hindered by limited knowledge on transmission dynamics and genetic evolution of ASFV. This study was conducted to determine the genetic variation and epidemiology of ASFV in selected countries of eastern and southern Africa. This study used a multidisplinary approach involving phylogeographic dispersal analysis, partial and complete genome analysis to elucidate the transmission dynamics and genetic structure of the ASFV in eastern and southern Africa. The findings of this study highlight the high genotypic variability in ASFV strains circulating between Tanzania and its eight neighboring countries with several transboundary spread events. Among 15 different ASFV genotypes circulating between Tanzania and its eight neighboring countries, 13 were isolated either from warthog or ticks demonstrating the importance of ASFV sylvatic cycle in these countries.

This study showed that the ASFV genotype II which was not previously described in Malawi was present and causing recent ASF outbreaks in the country. Interestingly, the ASFV genotype II that caused an outbreak in Malawi during 2019 was genetically closely related to ASFV strains belonging to genotype II previously described in Tanzania, Zambia, Mozambique, Zimbabwe, Georgia, China, Vietnam, Estonia, Moldova, Czech Republic, Belgium, Poland and Russia. These findings suggest the regional transboundary transmission of ASFV and long distance transmission of this ASFV genotype II across different continents.

The results of this study confirmed the ASF outbreak in Rutana region in South-eastern Burundi and the responsible ASFV strain belonged to genotype X with high nucleotide identity to other ASFV genotype X previously described in Burundi, DRC, Tanzania and

Kenya. The virus showed high genetic similarities with ASFV strains previously described in domestic pigs, warthogs, and soft ticks in East African countries, indicating a possible common wild source and continuous circulation in domestic pigs in the region.

This is the first study to report the complete genome sequences of ASFV in Burundi and ASFV genotype II in Malawi. The complete nucleotide sequences of ASFV that caused the 2018 outbreak in Burundi and the virus responsible for the ASF outbreak in Malawi during 2019 showed more than 99% nucleotide identity to ASFV strains previously described belonging to genotypes X and II, respectively. Phylogenetic analysis based on ASFV complete genomes showed that the viruses described in this study were closely related to viruses previously described not only in eastern and southern Africa but also in Europe and Asia for the virus from Malawi. A high genetic variation was observed within the *EP402R* gene that encodes the CD2v major ASFV antigen protein, and the virus responsible for the 2018 outbreak in Burundi clustered into the ASFV serogroup 7 while the virus responsible for the 2019 outbreak in Malawi clustered into ASFV serogroup 8.

3.3 Recommendations

Based on the findings of this study, the following recommendations can be made:

a) In order to get the complete scenario of the ASFV transmission in eastern and southern Africa, more research on the sylvatic cycle of ASFV is recommended to understand the role of wild suids present in wildlife protected areas and that of the soft ticks inhabiting their burrows in the epidemiology of ASF at the regional level.

b) Antigenic characterization and complete genome sequencing of other ASFV strains circulating in different countries of eastern and southern Africa are recommended to fully understand molecular epidemiology of ASFV in these countries.

c) Further research on the impact of the genetic variation detected in viruses described in this study on the phenotypes of ASFV is recommended.

d) A regional and international approach is recommended to control the transboundary and long distance spread of ASF.

e) An ASF regional control programs need to be put in place to control the occurrence of ASF in the region.

f) An education program targeting all stakeholders involved in the domestic pigs' value chain should be implemented to increase awareness on biosecurity measures required to avoid farm to farm transmission of ASFV during an outbreak.

g) The results of this study are important in order to understand the transmission dynamics and genetic evolution of ASFV in eastern Africa and will serve as backbone for possible future investigations concerning the development of ASF effective control and prevention strategies, including vaccine, diagnostic test and antiviral treatment development.

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