Mosquitoes of the Namibian Zambezi Region and their associated viruses

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Statement of Authorship

I hereby confirm that this dissertation is solely my own work. I declare that any sources and references that have been used have been acknowledged and fully cited.

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(Place, date) **(Signature)**

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Scientific chapters and author contributions

This work was based on the following manuscripts:

Chapter I:

Guggemos HD, Fendt M, Hieke C, Heyde V, Mfune JKE, Borgemeister C, Junglen S. **Simultaneous circulation of two West Nile virus lineage 2 clades and Bagaza virus in the Zambezi region, Namibia.** PLoS Negl Trop Dis. 2021 Apr 2;15(4):e0009311. doi: https://doi.org/10.1371/journal.pntd.0009311. PMID: 33798192; PMCID: PMC8046352.

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Guggemos HD, Fendt M, Hermanns K, Hieke C, Heyde V, Mfune JKE, Borgemeister C, Junglen S. **Orbiviruses in biting midges and mosquitoes from the Zambezi region, Namibia.** J Gen Virol. 2021 Sep;102(9). doi: https://doi.org/10.1099/jgv.0.001662. PMID: 34554079.

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Summary

A large diversity of viruses is transmitted by hematophagous mosquitoes. Among them are several infectious agents of major concern for human health, like dengue virus, chikungunya virus, Zika virus, yellow fever virus, Japanese encephalitis virus, and West Nile virus. Apart from arboviruses that infect vertebrate hosts, mosquitoes also harbor a range of insect-specific viruses that can have an influence on the transmission of other mosquito viruses.

In this work, a baseline study was conducted on the mosquitoes of the Namibian Zambezi Region and their accompanying viruses. Mosquito sampling was performed in National Parks and Conservancies across four different land-use types, including undisturbed habitats and areas used for agriculture or pasture. The mosquito community composition was investigated, and infection rates with arboviruses and insect-specific viruses were determined. Most sampling locations showed a high diversity of different mosquito species, which supported an equally high genetic diversity of RNA viruses from all the relevant families of mosquito viruses. Established arboviruses, like West Nile virus, were identified among the virus detections. Further, we could detect a high number of putatively novel viruses, most of which were mosquito-specific viruses from the viral families *Flaviviridae* and *Phenuiviridae*. No conclusive correlation could be derived between mosquito host biodiversity and RNA virus infection prevalence. Instead, mosquito community composition was found to have a significant impact on the infection prevalence of the viruses studied.

Zusammenfassung

Eine große Vielfalt von Viren wird durch hämatophage Stechmücken übertragen. Darunter finden sich mehrere Infektionserreger, die von großer Bedeutung für die menschliche Gesundheit sind, wie Dengue-Virus, Chikungunya-Virus, Zika-Virus, Gelbfieber-Virus, Japanisches-Enzephalitis-Virus und West-Nil-Virus. Neben Arboviren, die Wirbeltierwirte infizieren, beherbergen Stechmücken auch eine Reihe insektenspezifischer Viren, die einen Einfluss auf die Übertragung anderer Stechmückenviren haben können.

In dieser Arbeit wurde eine Basisstudie zu den Stechmücken der namibischen Sambezi Region und ihren Viren durchgeführt. Hierzu wurden in Nationalparks und Naturschutzgebieten Stechmücken aus vier verschiedenen Landnutzungstypen gesammelt, darunter ungestörte Lebensräume und landwirtschaftlich oder als Weideland genutzte Gebiete. Die Zusammensetzung der Mückengemeinschaft wurde untersucht und die Infektionsraten mit Arboviren und insektenspezifischen Viren wurden bestimmt. Die meisten Probenahmestellen zeigten eine hohe Diversität verschiedener Stechmückenarten, die eine ebenso hohe genetische Diversität von RNA-Viren aus allen relevanten Familien von Stechmückenviren aufwiesen. Unter den Virenfunden wurden auch etablierte Arboviren wie das West-Nil-Virus identifiziert. Darüber hinaus konnten wir eine große Anzahl mutmaßlich neuartiger Viren nachweisen, von denen die meisten stechmückenspezifischen Viren aus den Virusfamilien *Flaviviridae* und *Phenuiviridae* sind. Es konnte keine abschließende Korrelation zwischen der Stechmückenbiodiversität und der Prävalenz von RNA-Virusinfektionen abgeleitet werden. Stattdessen wurde die Zusammensetzung der Stechmückengemeinschaft als wesentlicher Einfluss auf die Infektionsprävalenz der untersuchten Viren identifiziert.

General Introduction

Arboviruses

The term “arbovirus” refers to viruses that are transmitted between hematophagous arthropod vectors and vertebrate hosts [1]. It is an acronym derived from “arthropod-borne virus” and was coined in the 1940s by the research team around Dr. William C. Reeves at the University of California at Berkeley when they were performing research on St. Louis and Western equine encephalitis viruses and their transmission by *Culex tarsalis* mosquitoes [2, 3]. In natural transmission cycles, arboviruses typically replicate in vertebrate hosts such as birds and small mammals, while humans and their farm animals usually represent dead-end hosts [4]. Apart from the horizontal transmission of arboviruses between vector and host, arboviruses can also be transmitted between female and male mosquitoes by venereal transmission and by vertical transmission from female mosquitoes to their offspring [5-7]. Infection with arboviruses can lead to severe disease in both humans and livestock [5]. Fueled by the Columbian Exchange, worldwide trade and travel, and the anthropogenic modification of primary ecosystems, particularly in the tropics, many arboviruses have spread over large parts of the globe in the last five centuries [8-10]. For example, the dengue virus (DENV) infects an estimated 500 million people each year on all continents except Antarctica [11, 12]. Most recently, DENV has extended its distribution to Europe, with autochthonous cases in France, Croatia, Portugal (Madeira), and Italy [13-17]. In comparison to most other arboviruses, DENV is able to use humans as an amplification host, enabling urban transmission cycles and driving urban outbreaks [5, 18]. The two major DENV mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, are among the most concerning arbovirus vectors [19, 20]. Apart from DENV, they are also the main vectors for chikungunya virus (CHIKV), Zika virus (ZIKV), and yellow fever virus (YFV), which are all three widely distributed and can cause large outbreaks with severe disease in humans [21-25]. *Aedes mcintoshi* and other floodwater mosquitoes within the genus *Aedes* are key vectors in the transmission of Rift Valley fever virus (RVFV) in Africa and the Arabian Peninsula [26-29]. Other important arbovirus vectors include species in the genus *Culex*, with the *Culex pipiens* mosquito complex responsible for West Nile virus (WNV) outbreaks in the Americas and Europe since the late 1990s, *Culex tritaeniorhynchus* transmitting Japanese encephalitis virus (JEV) in Asia, Australia, and Oceania, and species in the *Culex* subgenus *Melanoconion* involved in the transmission of Venezuelan equine encephalitis virus (VEEV) in Latin America [30-40].

Several important livestock pathogens, like the bluetongue virus (BTV), are transmitted by bloodsucking biting midges of the genus *Culicoides* [41].

Nearly all known arboviruses are ribonucleic acid (RNA) viruses; the African swine fever virus (ASFV), which is a double-stranded deoxyribonucleic acid (DNA) virus, represents a single exception [42, 43]. The arboviruses with an RNA genome belong to eight viral families: *Flaviviridae*, *Reoviridae*, *Togaviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Nairoviridae*, *Rhabdoviridae*, and *Orthomyxoviridae* [44-51]. RNA viruses do not have a proofreading activity, and thus, viral genome replication can result in error rates as high as 10^{-3} to 10^{-5} per nucleotide (nt) [52]. During infection, RNA viruses produce viral variants, described as viral “quasispecies” or “mutant swarms” [53]. This high genetic diversity of an RNA virus population is believed to enable RNA viruses to adapt to changing environments, including new vector or host species [54], and to represent emerging viruses [55]. Due to their relatively fast adaptation rate, their widespread distribution, and their significant impact on human and animal health, arboviruses pose a threat to global health [56-59].

Flaviviruses

The genus *Flavivirus* currently holds 53 classified species and is the largest genus within the family *Flaviviridae* [46, 60]. The majority of flaviviruses are arboviruses, with DENV, WNV, YFV, JEV, and ZIKV representing the most prominent members [61]. From the 15th to the 19th centuries, the name-giving type species YFV was responsible for large outbreaks with high mortality rates in port cities around the world, and starting in the 17th century, it ravaged the population of Latin America [62-65]. While the majority of flaviviruses are endemic to tropical areas, some species like WNV, JEV, and tick-borne encephalitis virus (TBEV) are also found in temperate regions [61, 66]. Up to date, highly effective and safe-to-use vaccines are only available for YFV, JEV, and TBEV [61, 67, 68].

Flaviviruses form enveloped virions of spherical shape with imperfect icosahedral symmetry [69]. Their genome is constituted by a single positive-sense RNA molecule, roughly in the range of 9,000–13,000 nt, with one single open reading frame (ORF) [46]. The 5’ untranslated region (UTR) of flaviviruses is approximately 100 nt in length and headed by a type I cap that is made up of a N^7 methylated (me^7)-guanosine cap

structure (5'-5' linked me^7 guanosine structures, me^7-GpppN-me^2) [70]. This 5' cap braces the viral RNA against 5'-exonuclease activity and has an important role in the translational initiation of the viral polyprotein [71, 72]. Following the cap structure, the flavivirus genome starts with a strictly conserved 5'-AG dinucleotide motif [73]. The following part of the 5' UTR forms two stem-loop structures, which are essential in the replication of viral RNA and the translation of viral proteins [74, 75]. The 3' UTR of flaviviruses ranges in length from 400–700 nt and is divided into three domains [76]. It contains a range of stem-loop structures, which are important for viral RNA synthesis and protection against exoribonuclease activity [77, 78]. The flaviviral 3' UTR does not contain a poly-A tail but ends with a strictly conserved CU-3' dinucleotide motif [73]. The 5' and 3' UTRs furthermore harbor elements that enable the cyclization of the viral genome, which is essential for the initiation of RNA synthesis [79, 80]. The flaviviral ORF encodes a single precursor polyprotein, which is processed by proteolytic cleavage into seven nonstructural (NS1, NS2A/B, NS3, NS4A/B, NS5) and three structural proteins (E, prM, C) [46, 81]. The nonstructural proteins NS3–an RNA helicase and triphosphatase–and NS5–an RNA-dependent RNA polymerase (RdRP) and N-terminal methyltransferase–are responsible for the majority of viral enzymatic activity [82].

Orbiviruses

The genus *Orbivirus* in the family *Reoviridae* currently holds 22 approved species [83]. The majority of them are arboviruses, with bluetongue virus (BTV), African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV), equine encephalosis virus (EEV), and Palyam virus (PALV) being the most important veterinary pathogens [84]. These five orbiviruses are all transmitted by biting midges of the genus *Culicoides* [85-90]. Disease in humans that is caused by an infection with members of the genus *Orbivirus* has so far only been reported in very rare cases [91]. The type species BTV is responsible for epidemic outbreaks of bluetongue disease in sheep and cattle, documented in South Africa since the late 18^{th} century [84]. Nowadays, orbiviruses are globally distributed, with BTV present on all continents except for Antarctica and EHDV also occurring on many continents [92]. Commercially available vaccines against BTV, AHSV, and EHDV exist, but none of them is both

highly effective and safe to use, as high serotype variations make vaccine development very difficult [93-99].

Orbiviruses form nonenveloped virions of icosahedral shape with a three-layered shell approximately 90 nm in diameter [100]. Their genome consists of ten segments of double-stranded RNA (dsRNA), which are named according to their nucleotide length in decreasing order, S1 to S10 [101]. During virus particle formation, one copy of each of these genome segments is incorporated into the capsid at the genome packing stage [102]. When two different orbiviruses co-infect a host, they can exchange entire genome segments through a process called reassortment [103]. The total genome size of orbiviruses is around 19,000 nt, with the tick-borne orbiviruses having slightly smaller genomes than the insect-borne orbiviruses [104].

The different orbivirus genome segments harbor UTRs of varying lengths that fulfill various functions in the viral life cycle. The UTRs of the BTV segment S10, for example, initiate RNA packing in the formation of virus particles [105]. The 5'-UTR of most orbiviruses starts with a 5'-GU dinucleotide motif, and the 3'-UTR does not contain a poly-A tail but usually ends with an AC-3' dinucleotide motif [84]. The orbivirus genome codes for at least eleven different proteins, seven structural (VP1-VP7) and four non-structural (NS1-NS4) [100, 106]. The structural proteins VP1 (Pol), VP4 (Cap), and VP6 (Hel) also have an important role in the enzymatic processing of RNA, while the VP2 (OC1), VP3 (T2), VP5 (OC2), and VP7 (T13) proteins form the shell layers of the mature virions [100].

Insect-specific viruses

Insect-specific viruses (ISVs) cannot replicate in vertebrates and are sustained in an insect-only transmission cycle [107]. The first ISV was discovered in 1975 in an *Aedes aegypti* embryonic cell line and named Cell-Fusing Agent virus (CFAV) as it induced the fusion of infected mosquito cells [108, 109]. Several years later, it was found to belong to the genus *Flavivirus* [110]. Due to advancements in Next Generation Sequencing (NGS) techniques and intensified mosquito sampling efforts, many additional ISVs have been discovered in recent years [111]. ISVs show a worldwide distribution and are found in many RNA virus families, including *Flaviviridae*,

Peribunyaviridae, *Phenuiviridae*, *Rhabdoviridae*, *Mesoniviridae*, *Togaviridae, and Reoviridae* [60, 112-114].

In vertebrate cells, the replication of ISVs is restricted at several stages, including virus attachment and entry, RNA replication, and the assembly and release of virions [115-119]. The full process remains to be elucidated, but physiological temperature seems to play a role, as ISVs are not able to replicate at the higher temperatures occurring in vertebrates [119, 120].

In contrast to vertebrates, insects lack an adaptive immune system [121]. Instead, they rely on an innate immune system that is mostly based on RNA interference (RNAi) as a mode of action [122]. Other factors include phagocytosis, encapsulation, nodulation, and various signaling pathways leading to the secretion of effector molecules, such as reactive oxygen species (ROS) and antimicrobial peptides (AMPs) [122]. In addition, the midgut microbiome of insects was shown to have antipathogenic features, such as the production of antiviral secondary metabolites [123-125]. All these mechanisms help insects defend themselves against viral pathogens, however, an elimination of an infection is mostly not possible, leading to long-lasting or persistent infections and thus allowing for lifelong transmission of viruses [126]. Although these insights have mainly been obtained from studies on arboviruses, more recent studies show a similar extensive activation of RNAi-mediated immune response for ISVs [127-131].

The transmission of ISVs is not yet very well understood. Three routes have been described for transmission between insects: a vertical route from parents to offspring, a less common horizontal venereal route between mating partners, and a horizontal oral route during feeding [132-138].

ISVs can influence the susceptibility of insects to secondary infections with other ISVs or arboviruses [139]. In co-infected or superinfected insects, this can either have a suppressing or an enhancing effect on arbovirus transmission, replication, and dissemination [140-145]. One of these effects is called “superinfection exclusion”. In this case, pre-infection of cells with an ISV, as demonstrated for Eilat virus (EILV; family *Togaviridae*, genus *Alphavirus*), inhibits subsequent infection with closely related viruses (homologous interference), as well as divergent viruses (heterologous interference) [114, 115, 141].

Dilution effect and amplification effect hypothesis

The dilution effect and amplification effect hypotheses both address the influence of biodiversity on the emergence of infectious disease [146]. According to the dilution effect hypothesis, an increase in the biodiversity of an ecological system, e.g., an increase in vertebrate diversity, leads to a decline in the transmission of infectious disease as infected and amplifying hosts are less likely to come together [147-150]. The amplification effect hypothesis assumes that an increase in the biodiversity of an ecological system leads to an amplification in the transmission of infectious disease [150]. For example, this could be the case when more competent hosts are present in more biodiverse ecosystems or when the transmission of pathogens is density-dependent [148, 149, 151, 152]. There is evidence that both of these effects can be observed in nature, although findings in agreement with the dilution effect hypothesis are more common [153-157]. The dilution effect hypothesis has prominently been studied in experiments on Lyme disease, a tick-borne bacterial disease that infects humans and is widespread in the northeastern USA [158]. It was shown that, under certain conditions, the survival of tick vectors could be reduced by a higher diversity of the host community [146, 159, 160]. However, studies on malaria prevalence and primate hosts found evidence for the amplification effect [161]. In some systems, both the dilution and amplification effects seem to exist, and the overall impact (amplification, dilution, or no net effect) is a consequence of the magnitude of the individual effects [162].

Social-ecological transformation in the Namibian Zambezi Region

The term “social-ecological system” was first mentioned by Everett Ratzlaff in 1969 [163, 164]. According to Berkes and Folke, it refers to a system in which social (human) and ecological (biophysical) subsystems are combined to create an integrated perspective of “humans in nature” [165, 166]. Consequently, social-ecological transformation deals with the transformation of the human system on one side and the transformation of the ecological system on the other side, as well as all the interactions between these systems.

The Zambezi Region is situated in the most northeastern part of Namibia’s national territory. Its vegetation, dominated by broadleaved woodland savanna and grassland floodplains, fosters a terrestrial biodiversity that is among the highest of all parts of

Namibia [167, 168]. A large part of the region is under natural protection. Around 28% of its total area is covered by three state-run National Parks, and about 27% is occupied by 15 Conservancies, which practice community-based natural resource management (CBNRM) [169]. These Conservancies and National Parks harbor a large number of lodges and campsites, which together with the trophy hunting industry make up an important part of the local economy [170].

Despite abundant natural resources and notable economic progress, all southern African countries, including Namibia and the Republic of South Africa, are on the United Nations list of developing countries, with GDP per capita ranging from 449 US dollars (Mozambique) to 6405 US dollars (Botswana) in 2020 [171-173]. These are countries in which even today a very large part of the population is still mainly dependent on farming and subsistence farming (in Namibia, 70% and 23%, respectively) [174, 175]. In the Namibian Zambezi Region, like in many other parts of southern Africa, the production of livestock has always been regarded as a major stimulus for rural development, and the traditional communities usually rely on cattle husbandry as a base for their livelihoods [176-179]. Agriculture is usually performed on a small scale, apart from some large-scale development green schemes, like the Kalimbeza rice project and the Katima/Liselo farm project [178, 180, 181]. Wood logging often happens on an illegal level and is therefore not always performed in a controlled and sustainable way [178, 182-184].

In recent years, the region has been subject to fast-moving structural change, with increasing population growth rates [178]. The economic expansion is being fostered by the finalization of the Walvis Bay-Ndola-Lubumbashi Development Corridor (WBNLDC) in 2004, which further drives land use and land cover change in the whole region, thereby influencing biodiversity and species composition in the affected ecological habitats [178, 185-189].

Objective of this work

The aim of this study is to assess the diversity of mosquito species in the Namibian Zambezi Region and to characterize the mosquito viruses that accompany them.

Mosquitoes shall be collected in sampling locations in four different land-use categories, including pristine habitats and areas used for agriculture or pasture, in

Bwabwata and Mudumu National Parks as well as in Mashi and Wuparo Conservancies.

Mosquito species shall be identified morphologically, and the mosquito community composition of the different sampling sites is to be examined. The sampled mosquitoes shall be screened for viral infections, and detected viruses shall be characterized genetically and phylogenetically. Live virus isolates shall be derived for selected viruses and characterized in cell culture experiments. Virus community composition and infection prevalence in the sampled mosquitoes shall be examined for the different sampling sites. Based on this data, the connection between virus infection prevalence, mosquito community composition, and land-use type shall be studied. Particularly, a possible effect of land-use change on mosquito community composition and virus infection prevalence is to be investigated.

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Chapter I: Simultaneous circulation of two West Nile virus lineage 2 clades and Bagaza virus in the Zambezi region, Namibia

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Abstract

Flaviviruses include a great diversity of mosquito-borne arboviruses with epidemic potential and high global disease burden. Several flaviviruses are circulating in southern Africa affecting humans and livestock, among them West Nile virus (WNV) and Wesselsbron virus. Despite their high relevance, no arbovirus surveillance study has been conducted for more than 35 years in Namibia. In this study we assessed the diversity of flaviviruses circulating in mosquitoes in the densely populated, semi-tropical Zambezi region of north-eastern Namibia. In total, 10,206 mosquitoes were sampled in Bwabwata and Mudumu national parks and Mashi and Wuparo conservancies and screened for flavivirus infections. A high infection rate with insect-specific flaviviruses was found with 241 strains of two previously known and seven putative novel insect-specific flaviviruses. In addition, we identified ten strains of WNV in the main vector *Cx. univittatus* sampled in the Mashi conservancy. Surprisingly, the strains fell into two different clades of lineage 2, 2b and 2d. Further, three strains of Bagaza Virus (BAGV) were found in *Cx. univittatus* mosquitoes originating from Mudumu national park. Assessment of BAGV growth in different cell lines showed high replication rates in mosquito and duck cells and about 100,000fold lower replication in human, primate and rodent cells. We demonstrate a wide genetic diversity of flaviviruses is circulating in mosquitoes in the Zambezi region. Importantly, WNV and BAGV can cause outbreaks including severe disease and mortality in humans and birds, respectively. Future studies should focus on WNV and BAGV geographic

distribution, as well as on their potential health impacts in and the associated social and economic implications for southern Africa.

Author summary

Mosquitoes serve as vectors for the transmission of infectious diseases. Some of the most important mosquito-borne arboviruses belong to the genus *Flavivirus*, which can induce severe disease in humans and livestock. Surveillance of vector populations provide information on circulating arboviruses and may help to identify local outbreaks. Here we sampled mosquitoes over three wet seasons in the densely populated, semi-tropical Zambezi region of north-eastern Namibia and tested them for infections with flaviviruses. We observed simultaneous circulation of two different West Nile virus clades in the main vector species *Cx. univittatus*. Humans infected with West Nile virus can develop flu-like symptoms or in rare cases meningoencephalitis. Further, we detected Bagaza virus in *Cx. univittatus* from another locality and season. Bagaza virus infects birds leading to high mortality rates and may also infect humans. Our data suggest that both viruses are endemic in the Zambezi region and may affect human health and well-being in Namibia.

Introduction

The genus *Flavivirus* within the family *Flaviviridae* contains some of the most dangerous and life-threatening mosquito-borne viruses like Dengue virus, Yellow fever virus, Japanese encephalitis virus, Zika virus and West Nile virus (WNV) [1]. Land use and socio-economic changes, as well as global trade and travel fueled the spread of these and other flaviviruses to new geographic regions where they caused epidemics and often became endemic [2]. For instance, Dengue virus is now endemic in >100 countries and affects about 400 million people each year [3].

Several flaviviruses are endemic in southern Africa, with WNV and Wesselsbron virus (WSLV) among the most prevalent ones [4]. WNV is a mosquito-borne flavivirus that was first isolated in 1937 from a patient with febrile symptoms in the West Nile district of Uganda [5]. The virus is maintained in nature in a mosquito-bird transmission cycle where it can be transmitted to humans and horses by a bite of an infected mosquito. WNV is geographically the most widely distributed encephalitic flavivirus and has caused outbreaks in the Americas, Africa, Europe and Asia [6-10]. Approximately 80%

of human infections with WNV are asymptomatic. Symptomatic patients usually show flu-like symptoms, such as fever, fatigue, headache, nausea, muscle pain and rash [11, 12]. Less than 1% of WNV patients develop severe symptoms, such as meningoencephalitis that can lead to death [13]. WNV can cause sporadic outbreaks, usually after heavy rainfall, resulting in a sharp vector population build-up [14]. In 1974, the Karoo and Northern Cape provinces in South Africa experienced a large WNV outbreak that affected ten thousand of humans though no cases of encephalitis and no deaths were recorded [15-17]. In South Africa WNV infections have been observed in a range of wild and domesticated animals and seroprevalence rates of 24%, 18% and 30% against WNV were detected in humans, cattle, and sheep, respectively using an *in-house* ELISA indicating that flaviviruses are highly prevalent across the country [18, 19].

For WSLV, first isolated in the South African town of Wesselsbron in 1955, similar seroprevalence rates in humans and cattle were observed in South Africa [18, 20]. WSLV causes arthralgia, myalgia and fever in humans [21, 22]. Symptoms in cattle and sheep are more severe and similar to Rift Valley fever with abortion rates of up to 20% [23-25].

Bagaza virus (BAGV) is a mosquito-borne flavivirus pathogenic to birds and has only recently been detected in dead monal pheasants near Pretoria in South Africa [26]. Bagaza virus shares high sequence similarity with Israel turkey meningoencephalitis virus (ITV), which was first isolated 1958 from domesticated turkeys in Israel [27] and was detected in South African turkeys in 1978 [28]. Based on the high sequence identity, both viruses have been suggested to belong to the same species [29]. Typical symptoms in susceptible bird species include weight loss, weakness, and apathy with mortality rates as high as 30% in partridges and pheasants [30, 31]. BAGV was first isolated in 1966 from *Culex* mosquitoes collected in Bagaza, Central African Republic [32]. Subsequently, it has been detected in mosquitoes collected in Senegal and Mauritania [33-35] and India [36]. BAGV neutralizing antibodies were later detected in encephalitic patients from India suggesting that the virus can also infect humans [36]. BAGV was first isolated from vertebrates in 2010 from dead red-legged partridges and pheasants in southern Spain [31].

Usutu virus (USUV) and Banzi virus (BANV) are also endemic in the region and were both first isolated close to the Usutu river in South Africa [37-39]. Finally, there are historic reports of DENV epidemics in southern Africa in the late 19th and early 20th

century, with most recent reports from northern Mozambique dating back to 1984 [40]. Since then, only few reports on DENV in the region have been published, primarily originating from imported cases, and hence DENV is most likely not circulating in this part of the continent [41].

Little is known on the current incidence of flaviviruses in Namibia. WNV, WSLV, BANV, Spondwenni and Yellow fever have been detected in the local population in serosurveys across the north-eastern Zambezi region in the 1950s and 1960s with antibody prevalence rates of 12%, 23%, 9%, 4 and 2% in neutralization assays [42]. In the 1980s, rates of 29% WNV and 5% WSLV seroprevalence were recorded by hemagglutination inhibition assays in the same region [43], and WNV was detected in a febrile patient in Namibia's Ovamboland in 1989 [44]. Since independence in 1990, only one mosquito-borne arbovirus surveillance study was conducted in Namibia, focusing on the hot and semi-arid central region of the country, with a seroprevalence rate not exceeding 8% against flaviviruses reported in human sera by ELISA testing [45]. Yet since the 1980s, no comparable data has been gathered for the rather densely populated, semi-tropical regions in the north of the country [45].

In this study we sought to assess the diversity of flaviviruses circulating in mosquitoes in the Zambezi region in north-eastern Namibia. To obtain an overview on the genetic diversity of endemic viruses, we sought to sample a broad diversity of mosquito species from various land-use types.

Material and Methods

Ethics statement

A research permit to conduct this research was received from the National Commission on Research, Science and Technology (permit number RPIV00442018).

Mosquito sampling and identification.

Mosquitoes were sampled in north-eastern Namibia in the Sachinga Livestock Development Centre in May and June 2018, in Mudumu National Park and Wuparo Conservancy in November and December 2018, in Bwabwata National Park in February and March 2019, and in Mashi Conservancy in March and April 2019 and in February and March 2020 (**Fig. 1**). Four sites each in the National Parks were selected to represent sites with medium and high pressure of elephants. In the conservancies,

we selected four sites each in rangeland, agricultural field and in the conservancy core area to cover all existing land-use types of the conservancies. In total, two BG Sentinel traps (Biogents AG, Regensburg, Germany), two CDC Light traps and one CDC Gravid trap (both John W. Hock Company, Gainesville, United States of America) were operated from dusk till dawn for five consecutive days at each site (total trapping effort 1,480 trap nights). BG Sentinel traps were baited with BG-Lure (Biogents AG, Regensburg, Germany), 1-Octen-3-ol and worn socks on different days. The combination of trap types and attractants was used in order to sample a high diversity of mosquito species. Mosquitoes were stored in liquid nitrogen in the field. Mosquito species were identified morphologically in the laboratory using standard keys [46-48].

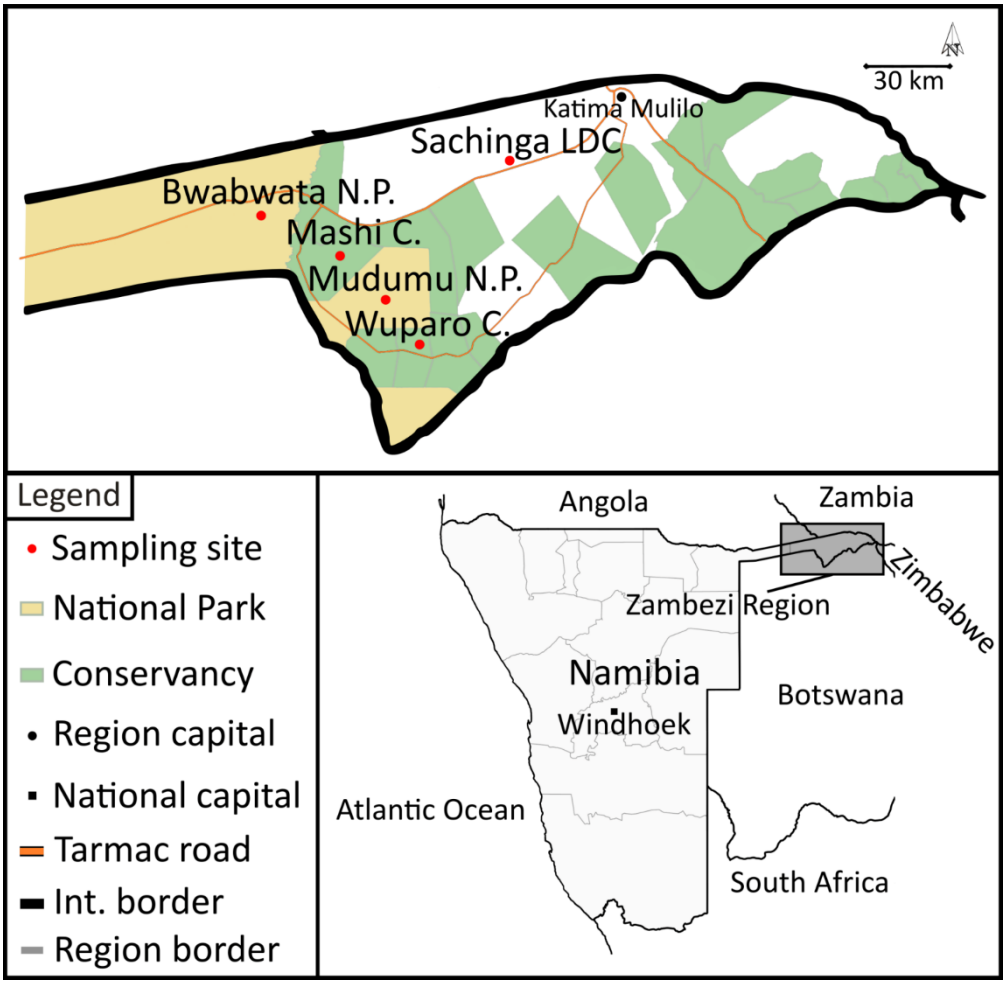

Fig 1. Schematic map of mosquito-sampling sites in Namibia. Samples were retrieved from Bwabwata and Mudumu National Parks, Wuparo and Mashi Conservancies and Sachinga Livestock Development Centre (Sachinga LDC). The map base layer is based on https://www.naturalearthdata.com, and the map's details are based on [49].

Viral RNA Extraction and PCR-screening.

Individual mosquito specimens were homogenized in 500 µl phosphate-buffered saline (PBS) using ceramic beads and a Tissue Lyser (QIAGEN, Hilden, Germany) and subsequently arranged into pools of 10 according to species and sampling location using 100 µl of the individual mosquito homogenate. The rest of the mosquito homogenate was retained for further analysis based on individual specimens. RNA was extracted from 200 µl of pooled mosquito homogenates using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics, Mannheim, Germany). The RNA was eluted in 100 µl of provided elution buffer. Subsequently cDNA synthesis was performed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific GmbH, Dreieich, Germany) and random hexamer primers (Integrated DNA Technologies Germany GmbH, Munich, Germany). Mosquito pools were screened for flaviviruses using generic PCR assays targeting the RNA-dependent RNA polymerase gene according to published protocols [50, 51]. Nucleotide sequences were obtained by Sanger sequencing (Microsynth Seqlab GmbH, Göttingen, Germany), analysed in Geneious 9.1.8 (Biomatters ltd., Auckland, New Zealand) and compared to GenBank using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, all mosquito pools were tested for WNV [52], Dengue virus [53], Zika virus [54], Yellow fever virus [55] and BAGV (forward 5'-TCCAGGGAAGACCAGAGAGG-3', reverse 5'-AGGCTTCAGCAATCCTTCCC-3', probe 5'-FAM-TGCGTTGAACACCTTTACCA-ZEN-3') using virus specific real-time-PCRs. RNA was extracted from single mosquito specimen of virus-positive pools and tested individually for infection using virus-specific RT-qPCRs as described above.

Genome sequencing and analysis.

A fragment of the viral E protein (1,336 nucleotides) of all detected WNV strains was amplified by nested PCR using the primers WNV-E F1 5'-GAGGGAGTGTCTGGAGCTAC-3', WNV-E R1 5'-CTGTCACGGGCATTGATTCC-3'; WNV-E F2 5'-TGGGTTGATCTGGTACTGGA-3', WNV-E R2 5'-GAAGTCCCTGTGTGATCCAG-3'. Full WNV genomes of two selected strains (M6848NA-2020 and M6646-NA-2020) were sequenced by amplifying overlapping PCR amplicons of 800-1,000 nucleotides in length using generic nested primers based on an alignment of WNV lineage 2 sequences (primer sequences will be published elsewhere). For full genome sequencing of the BAGV strain MP314-NA-2018 nested

primer pairs were designed based on an alignment of all currently available BAGV genomes aiming to amplify overlapping fragments of approximately 1,500 nucleotides (S1 Table). Amplicons were sequenced by Sanger sequencing and genomes were assembled using closely related strains as reference genomes in Geneious 9.1.8 (Biomatters ltd., Auckland, New Zealand).

Phylogenetic analyses.

Nucleotide and amino acid sequences of RdRp and E genes, as well as entire open reading frames (ORFs) were aligned with related viral sequences in Geneious using the CLUSTAL W [56] and MAFFT algorithms [57]. Phylogenetic trees were inferred by the maximum-likelihood (ML) method with the best suitable substitution matrix as identified by MEGA-X [58] using PhyML [59]. The phylogeny of all detected flaviviruses was inferred using the GTR model with a fixed Gamma shape parameter of 0.36 and an estimated fraction of invariable sites. The phylogenetic relationship based on WNV E genes was inferred with the GTR model using a fraction of 0.46 invariable sites and a fixed Gamma shape parameter of 1.2. The phylogenetic relationship based on WNV full genomes was inferred with the JC69 model using an estimated fraction of invariable sites and an estimated Gamma shape parameter. The BAGV phylogeny based on full BAGV genomes was inferred using the TN93 model, a fixed Gamma shape parameter of 0.27, and an estimated fraction of invariable sites and the BAGV tree based on RdRP sequence fragments was inferred with the TN93 model, a fixed Gamma shape parameter of 0.34, and an estimated fraction of invariable sites. Confidence testing was performed based on 1,000 bootstrap iterations.

Genetic barcoding for mosquito species identification and blood-meal analysis.

From selected mosquito specimens a fragment of the cytochrome c oxidase I (COI) gene was amplified based on generic primers for invertebrates [60]. To identify the vertebrate feeding source of selected mosquitoes, a fragment of the COI gene was amplified using generic vertebrate specific primers [61]. Obtained PCR-products were sequenced by Sanger sequencing (Microsynth Seqlab GmbH, Göttingen, Germany) and compared to GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/).

Virus isolation in cell culture.

For virus isolation attempts, mosquito (C6/36, *Aedes albopictus*; CXT, *Culex tarsalis)* and mammalian (VeroE6, African green monkey) cell lines were either inoculated with an aliquot of the virus-positive mosquito-pool or an aliquot of the individual virus-positive mosquito homogenate as described previously [62]. Cultures were checked daily for occurrence of a cytopathic effect (CPE). An aliquot of the supernatant (100 µl) was used for infection of fresh cells eight days post infection (passage 1). This procedure was repeated four times. The cell culture supernatants of each passage were in addition checked for viral replication by real-time PCR as described below.

Virus stocks were prepared from infectious cell culture supernatant of MP312-NA-2018 passage 4, MP314-NA-2018 passage 4, and MP370-NA-2018 passage 5. The number of infectious particles was determined by Tissue culture Infectious Dose 50 (TCID50) end-point dilution assay [63, 64].

Virus growth kinetics.

Different vertebrate (VeroE6, African green monkey; BHK-21, hamster; KN-R, cattle; DF-1, chicken [65]; LMH, chicken [66]; AGE1.CR, duck [67]; HEK293, human) and insect (C6/36, *Aedes albopictus*; CXT, *Culex tarsalis)* cell lines were infected in duplicates at a multiplicity of infection (MOI) of 0.1 and 0.01, respectively. Aliquots of cell culture supernatants were sampled every 24 hours for five days and virus copy numbers were determined by virus specific RT-qPCR using a plasmid-based standard dilution series. Briefly, the 114 nucleotide BAGV qPCR amplicon was amplified by conventional PCR using infectious supernatant of MP314-NA-2018 and subsequently cloned into the TOPO TA vector using the TOPO TA cloning kit according to the manufacturer's protocol (Thermo Fisher Scientific GmbH, Dreieich, Germany). An overnight culture of a positive clone was prepared in LB medium supplemented with ampicillin (100 µg/mL) and plasmid DNA was extracted with the QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany). Plasmid concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) and a tenfold serial dilution series from $1x10^1$ – $1x10^6$ genome copies/ml was used for quantification.

Results

Mosquito collection and flavivirus screening.

A total of 10,206 mosquitoes were collected in national parks and conservancies in the Zambezi region, Namibia, from May 2018 to March 2020 (**Fig 1**). In total, 42 different species were collected with *Culex univittatus* (3032, 30%), *Coquillettidia metallica* (1851, 18%) and *Mansonia uniformis* (1652, 16%) as the most abundant species. About three quarters of the sample set represented female (n = 7840, 76.8%) and about one quarter male mosquitoes (n = 2181, 21.4%) and for 185 (1.8%) mosquitoes the sex could not be identified due to poor sample condition. The average catch size was 4.3 mosquitoes per catch bag. Most of the mosquitoes were collected in the Wuparo conservancy and low catch rates were associated with low precipitation rates (**Fig 2**). After individual homogenization, all mosquitoes were sorted into 1,021 pools, comprising 10 individuals each.

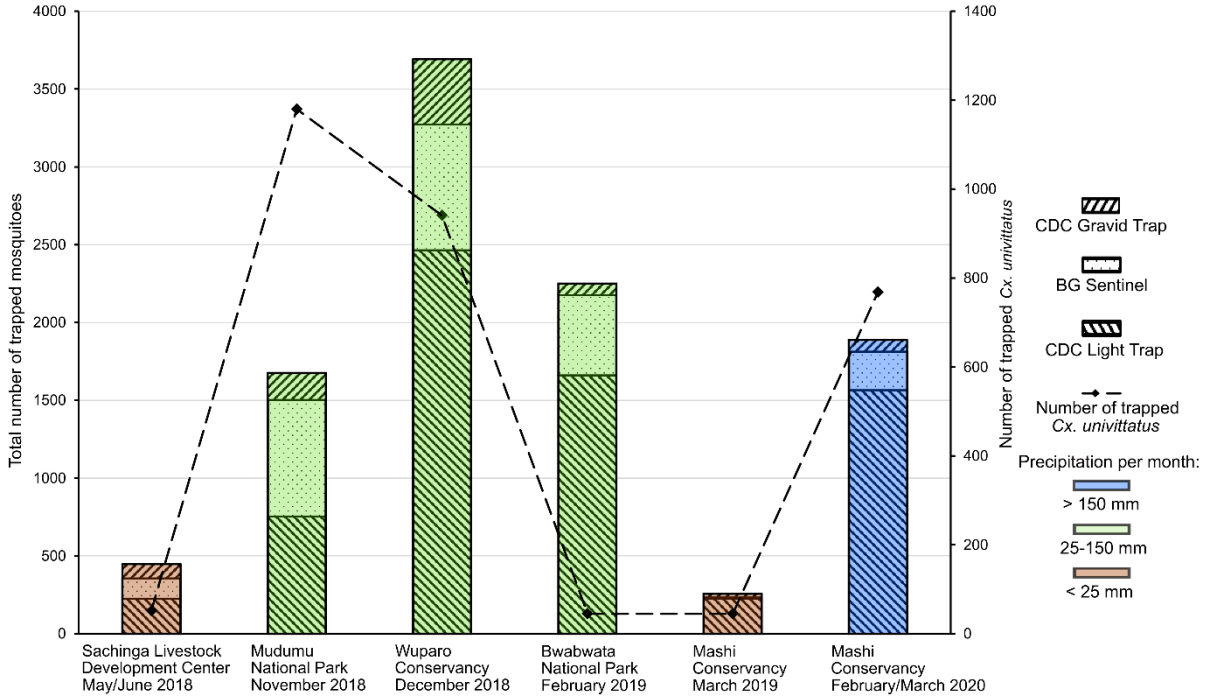

Fig 2. Overview of collected mosquitoes in the Zambezi region, Namibia. Number of sampled mosquitoes per locality and collection time is given by bar charts according to trap type and amount of rainfall at the time of collection [68]. Number of collected *Cx. univittatus* mosquitoes per locality is shown by a dashed line.

Screening of all pools with a generic flavivirus RT-PCR yielded 246 viral RdRP sequences falling in eleven clusters. Two sequences from two pools of female *Cx. univittatus* mosquitoes showed 96.9% and 97.9% identity to the South African WNV

strains HM147822 and EF429197, respectively, and three sequences from *Culex* sp. mosquitoes showed 99.3-100% identity to BAGV indicating the detection of WNV and BAGV in mosquitoes from Namibia. The term strain is in the following referred to as a variant of a given virus species. All other 241 sequences showed identities to insect-specific flaviviruses (ISF). We detected 10 strains of Culex flavivirus in *Culex* spp. mosquitoes and 60 strains of Mosquito flavivirus AMH010516 in *Mansonia* spp. and *Cx. univittatus* mosquitoes (**Table 1**). The other 171 sequences assembled in seven groups which showed pairwise identities of 70-77% to known ISF suggesting that they most likely belong to seven distinct species (**Table 1**). In addition, all mosquito pools were tested negative for DENV, ZIKV and YFV in specific qPCR assays.

Table 1. Detected insect-specific flaviviruses in the Zambezi region, Namibia.

Virus	Reference sequence	Mosquito host (number of detections)
Culex flavivirus	MP572-NA-2018	*Culex sp. (7),* *Cx. nebulosus (1),* *Cx. univittatus (1),* *Cx. quinquefasciatus (1)*
Mosquito flavivirus AMH010516	MP76-NA-2018	*Mansonia. uniformis (58),* *Ma. africana (1),* *Cx. univittatus (1)*
Unassigned	MP74-NA-2018	*Coquillettidia metallica (86),* *Cx. univittatus (1)*
Unassigned	MP7-NA-2018	*Cq. metallica (10)*
Unassigned	MP484-NA-2018	*Ma. uniformis (38),* *Ma. africana (2),* *Cq. metallica (1),* *Anopheles sp. (1),* *Not determined (1)*
Unassigned	MP583-NA-2020	*Anopheles sp. (1),* *Cx. univittatus (1)*
Unassigned	MP815-NA-2019	*Ma. africana (14),* *Ma. uniformis (5),* *Cq. fuscopennata (1)*
Unassigned	MP63-NA-2018	*Aedes sp. (3),* *Cx. univittatus (1),* *Not determined (2)*
Unassigned	MP565-NA-2018	*Not determined (3)*

Phylogenetic analyses based on one representative strain of every identified virus species and selected flaviviruses confirmed the identification of WNV, BAGV and nine ISFs (**Fig 3**). WNV M6646-NA-2020 and BAGV MP314-NA-2018 grouped together with Dengue virus 2 (NC001474) in a monophyletic clade comprising mosquito-borne flaviviruses in sister relationship to the clade of ISFs. The Namibian ISFs did not group according to their geographic origin, but rather formed host species associated clades.

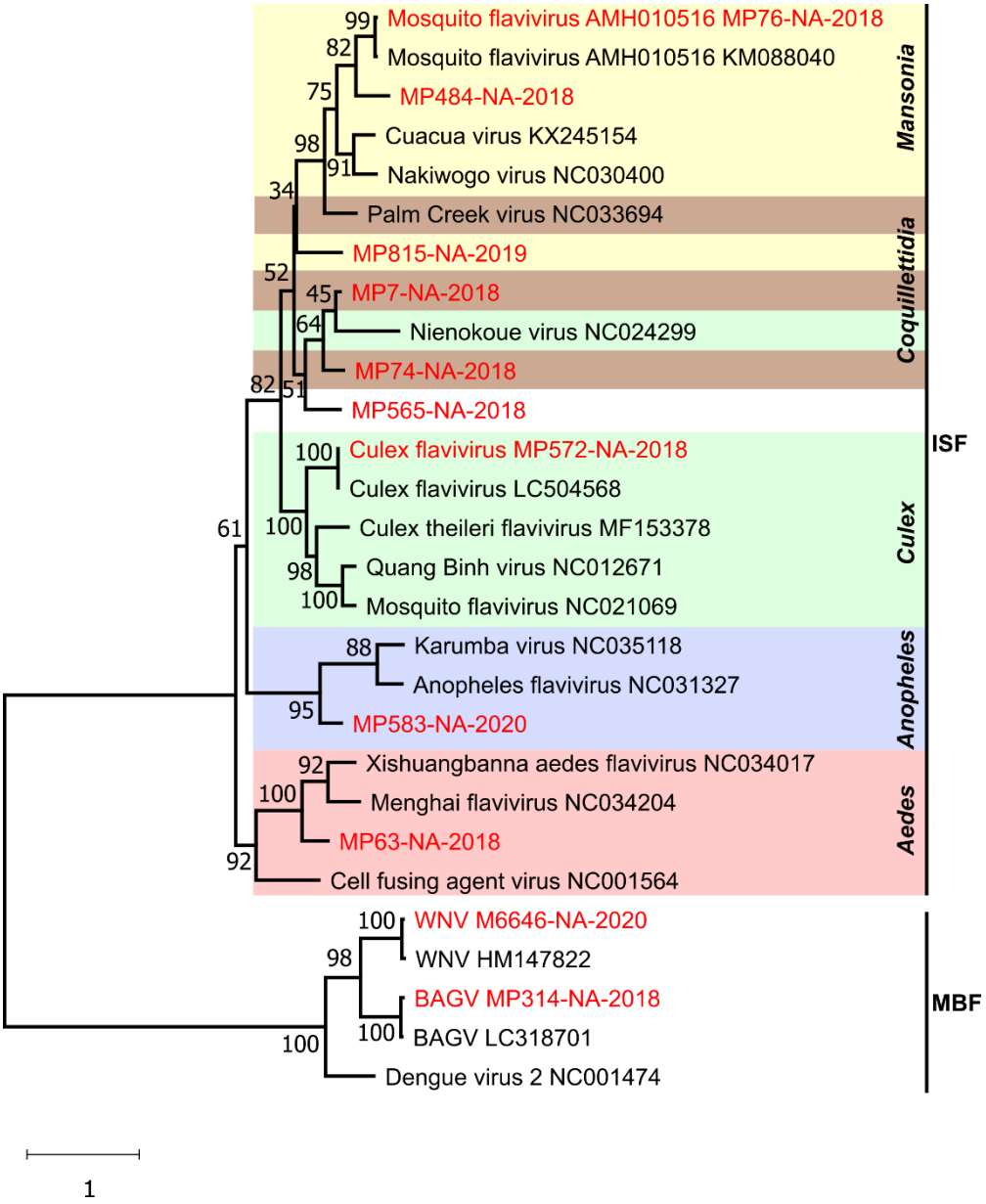

Fig 3. Phylogenetic relationship of flaviviruses detected in the Zambezi region. The phylogenetic tree is based on RdRP gene sequences of 153 nucleotides. Branch support is shown at each branch. Virus sequences detected in this work are shown in red. Gen Bank accession numbers are marked in grey.

Characterization of WNV.

Following the identification of WNV in two mosquito pools, all pools were screened for WNV infections by specific RT-qPCR and individual mosquitoes of the eight WNV-positive pools were tested by specific RT-qPCR. WNV was identified in ten individual female *Cx. univittatus* mosquitoes with viral genome copy numbers of 1.3 x 10^5 – 6.8 x 10^7 per mosquito (**Table 2**). All attempts to isolate the WNV strains from the pooled or individual mosquito homogenates in cell culture failed despite the detection of high viral loads in the mosquitoes. Mosquito species were confirmed by genetic barcoding. We further examined the WNV-positive mosquito extracts (n= 10) for remnants of vertebrate blood meal sources. We identified human mitochondrial DNA in the mosquito that was infected with WNV strain M6848-NA-2020. No vertebrate DNA was found in the other mosquitoes.

Table 2. WNV strains identified in the Mashi conservancy in this study.

WNV strain	Host	Locality	RNA genomic copies per mosquito	GenBank accession number
M6646-NA-2020	*Cx. univittatus*	Rangeland	7.94 10^6	MW383507
M6831-NA-2020	*Cx. univittatus*	Agricultural field	6.78 10^7	MW383509
M6848-NA-2020	*Cx. univittatus*	Agricultural field	1.20 10^9	MW383508
M7167-NA-2020	*Cx. univittatus*	Agricultural field	1.35 10^5	MW436414
M7170-NA-2020	*Cx. univittatus*	Agricultural field	1.01 10^7	MW436415
M7238-NA-2020	*Cx. univittatus*	Agricultural field	6.50 10^6	MW436416
M7302-NA-2020	*Cx. univittatus*	Agricultural field	5.86 10^7	MW436417
M7367-NA-2020	*Cx. univittatus*	Agricultural field	3.28 10^6	MW436418
M7373-NA-2020	*Cx. univittatus*	Agricultural field	8.9 10^6	MW436419
M7374-NA-2020	*Cx. univittatus*	Agricultural field	2.48 10^7	MW436420

E-gene sequence analysis and phylogenetic inference revealed a grouping of the ten identified WNV strains with lineage 2 sequences which widely occur across the African continent [69] (**Fig 4A**). Surprisingly, the detected WNV strains did not group together but fell within two different clades of WNV lineage 2. Nine of the ten WNV strains that originated from the agricultural field clustered with clade 2d, whereas strain M6646-NA-2020 that originated from rangeland grouped with clade 2b. Clade 2b has so far only been detected in South Africa in 1958 and in Cyprus in 1968 [69]. A complete genome of one strain from each clade was sequenced. Phylogenetic analyses based on complete coding sequences confirmed grouping with clades 2b and 2d and showed that M6848-NA-2020 and M6646-NA-2020 each were most closely related to viruses detected in South Africa (**Fig 4B**). Strain M6646-NA-2020 contained a deletion of 18 nucleotides in the 3'UTR region which was not present in any of the closely related WNV strains (**Fig 5**).

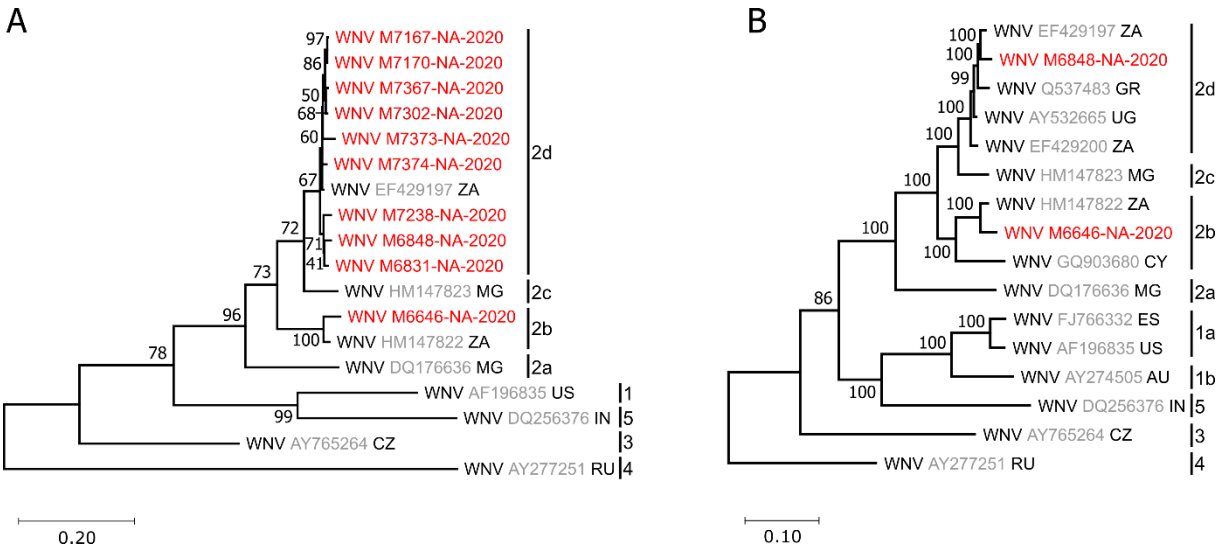

Fig 4. Phylogenetic relationship of detected WNV strains. The phylogenetic tree is based on a 1,032 nucleotide alignment of E-protein sequences of all detected WNV strains and selected global WNV strains (A) or on an alignment of the entire open reading frame (ORF) of WNV M6848-NA-2020 and M6646-NA-2020 and selected global WNV strains (B). Branch support is given for each branch. Virus sequences identified in this study are shown in red. Reference sequences are marked with their accession number in grey and the two-letter code of their countries of origin. AU, Australia; CY, Cyprus; CZ, Czechia; ES, Spain; GR, Greece; IN, India; MG, Madagascar; RU, Russia; UG, Uganda; US, United States of America; ZA, South Africa. The phylogenies are rooted to a WNV lineage 4 strain from Russia (AY277251).

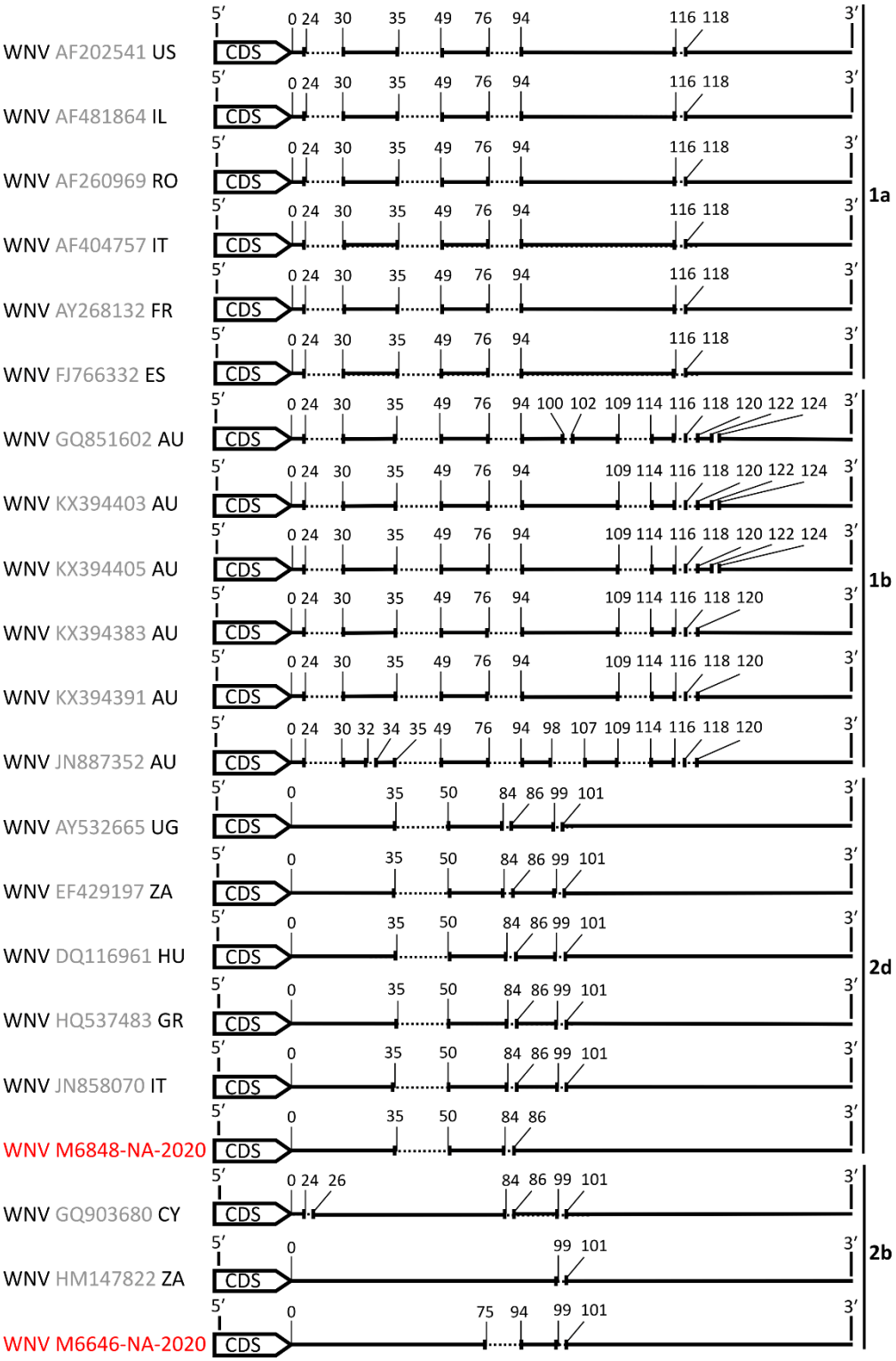

Fig 5. Schematic depiction of WNV 3' UTR sequences. Strains of different lineages were aligned, and insertions or deletions are shown. Numbers indicate genomic positions. Sequences identified in this study are shown in red.

Characterization of BAGV

DNA barcoding showed that the three BAGV-positive mosquito pools consisted of *Cx. univittatus* mosquitoes. Virus isolation attempts were successful for all three strains MP312-NA-2018, MP314-NA-2018, and MP370-NA-2018 in mosquito cells (C6/36). A CPE was observed in C6/36 cells, 24-, 10-, and 14-days post infection (dpi) for strains MP312-NA-2018, MP314-NA-2018, and MP370-NA-2018, respectively. Growth analyses in C6/36 cells revealed similar growth of the three strains and extremely high genome copy numbers of ca. $1x10^{12}$ RNA copies / ml five dpi (**Fig 6**). For further assessment of virus growth and putative host tropism, an array of different cell lines was infected with MP314-NA-2018. The mosquito cell line CXT was also permissive albeit genome copy numbers were approximately two log factors lower compared to C6/36 cells. Since BAGV is a bird pathogen, we infected three different avian cell lines. BAGV was only observed to replicate in AGE1.CR cells derived from muscovy ducks but not in LMH and DF-1 cells derived from chicken. Replication of BAGV in AGE1.CR was almost as productive as in C6/36 cells. BAGV growth was the least-well in mammalian cells with maximum genome copy numbers ca. 10^6-fold less than in C6/36 cells. However, similar viral RNA copy numbers were reached in non-human primate (Vero), human (HEK293T) and rodent (BHK-21) cells. No growth was observed in cattle-derived cells (KNR).

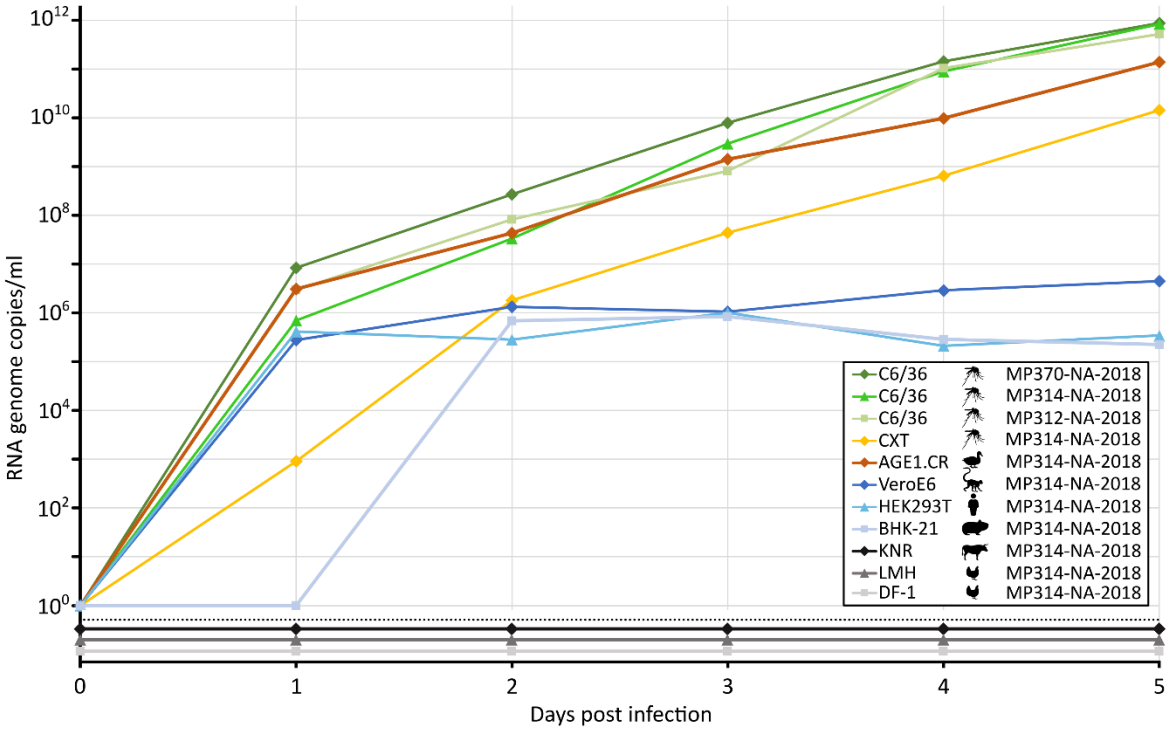

Fig 6. BAGV growth in vertebrate and insect cell lines. The mosquito cell line C6/36 (derived from *Aedes Stegomyia albopictus*) was infected with the three BAGV strains MP312-NA-2018, MP314-NA-2018, and MP370-NA-2018 in duplicates at an MOI of 0.01. Mammalian cell lines BHK-21 (hamster), HEK293T (human), VeroE6 (monkey) and KNR (cattle) were infected in duplicates with BAGV strain MP314-NA-2018 at an MOI of 0.1. Avian cell lines AGE1.CR (duck), LMH (chicken) and DF1 (chicken) and the insect cell line CXT (derived from *Culex tarsalis*) were infected in duplicates with BAGV strain MP314-NA-2018 at an MOI of 0.01. A sample of cell culture supernatant was taken every 24 hours for five consecutive days and viral copy numbers were determined by RT-qPCR.

The genome of BAGV strain MP314-NA-2018 was sequenced from infectious cell culture supernatant. The full sequence was uploaded to GenBank under accession number MW672101. Full genome based phylogenetic tree inference showed that BAGV MP314-NA-2018 was most closely related to BAGV strain LC318701 from Zambia (**Fig 7A**). In further phylogenetic analyses, which included the partial NS5 sequence of a BAGV strain (MN329584) that was isolated from monal pheasants in South Africa in 2016, BAGV MP314-NA-2018 grouped with this sequence (**Fig 7B**). As MP314-NA-2018 is clearly distinct from LC318701 from Zambia (97.5% divergence)

and from MN329584 from South Africa (99.2% divergence based on 1041 nucleotides of viral RdRP sequence), it seems to represent a strain endemic to Namibia.

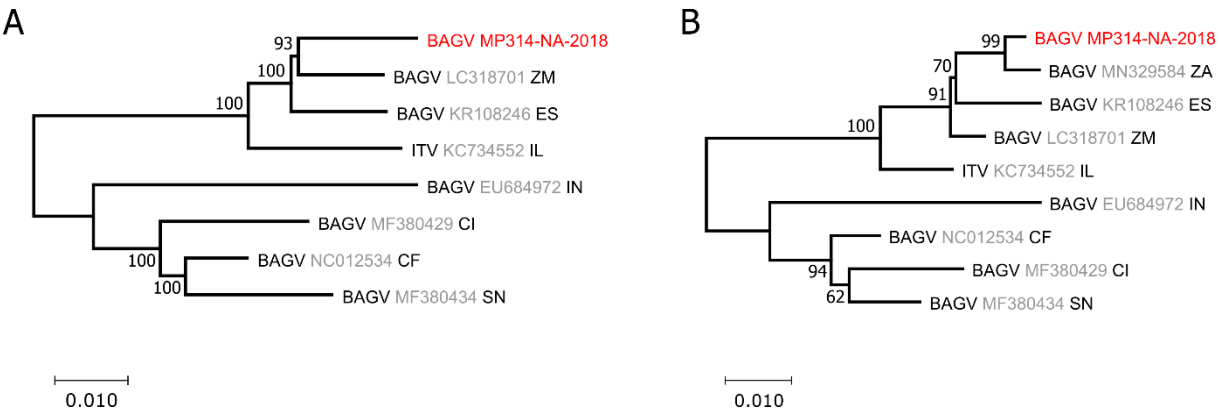

Fig 7. Phylogenetic inference of detected BAGV strains. Phylogenetic relationship of MP314-NA-2018, Israel turkey meningoencephalomyelitis virus (ITV) and selected global BAGV strains based on the entire RdRp gene (A). The tree is based on an alignment of 1,078 nt to include the partial NS5 sequence of a BAGV strain isolated from monal pheasants in South Africa in 2019 (B). Branch support is marked at each branch. Virus sequences detected in this work are shown in red. Reference sequences from GenBank contain the virus name, the accession number in grey and the two-letter code of their countries of origin. CF, Central African Republic; CI, Côte d'Ivoire; ES, Spain; IN, India; IL, Israel; SN, Senegal; ZA, South Africa; ZM, Zambia.

Discussion

Information on arbovirus circulation in vector populations in the tropics remain sparse, although surveillance in these regions is of particular importance since arboviruses are primarily endemic in tropical and subtropical regions, often causing local outbreaks there. Here, we screened mosquitoes from the Zambezi region of north-eastern Namibia for infection with flaviviruses and report the detection of the vertebrate-pathogenic arboviruses WNV and BAGV, as well as the identification of 241 strains of two previously known and seven putative novel insect-specific flaviviruses. This is the first detection of WNV and BAGV in insect vectors in Namibia and also the first detection of WNV in Namibia in more than 30 years.

We detected WNV at the end of the yearly rainy season in March 2020 but not at the end of 2018 nor during or at the end of the rainy season in 2019. Heavy precipitation is a key driver for an upsurge in mosquito populations and WNV infections, especially

in dryer landscapes where mosquitoes only find breeding places after heavy rainfall and in the wet season [70]. Such heavier rainfalls may have contributed to the detection of WNV in 2020. With 253.9 mm precipitation in the Zambezi region was notably higher between January and March 2020 compared to the 98.3 mm and 76.1 mm recorded for the same time period in 2018 and 2019, respectively [68].

WNV is transmitted by a large variety of mosquito species [71]. Mosquitoes of the *Culex pipiens* L complex are the primary WNV vectors in North America and Europe. In contrast, *Cx. univittatus* has been identified as the most important WNV vector in South Africa [72]. We exclusively detected WNV in female *Cx. univittatus* mosquitoes, confirming the importance of this species as WNV vector in southern Africa. Of note, WNV was only found in the Mashi conservancy in 2020 but not a single WNV infection was detected in Mudumu National Park and Wuparo Conservancy in 2018 where the absolute numbers of collected *Cx. univittatus* mosquitoes peaked (**Fig 2**). Such a discrete temporal and spatial distribution pattern of WNV has been observed in long term surveys in the USA [73].

We observed a WNV prevalence of 0.1% (10/10,206 mosquitoes). Other studies conducted in South Africa between 1956 to 1980 found WNV infection rates in mosquitoes of less than 0.026% [72]. However, infection rates in *Cx. univittatus* can reach up to 39.0/1000 or 9.6/1000 during WNV epizootics in the same region [72]. Notably, all the ten detected WNV strains fell into two different lineage 2 clades, with one strain grouping with clade 2b and nine strains grouping with 2d. The 2b strain (M6646-NA-2020) originated from rangeland and the 2d strains from agricultural fields in the Mashi conservancy that were about 9 km apart from each other, further emphasizing that WNV is endemic and diversified in Namibia. Identity values of 95.9–99.9% in pairwise alignment of partial E-protein fragments of the nine 2d sequences support a genetically diversified set of strains. This becomes particularly evident when comparing the observed genetic divergence rate of maximal 4% to that found in other studies. For example, nucleotide divergences in mosquitoes were found to range between 1-2% in the Montréal area of Canada from 2004-2016 [74], and below 1% in the US, 0.41%–0.72% in Texas [75], 0.26%–0.5% in Chicago [76], and 0.3% in Connecticut [77]. Higher divergence rates of 0.16-3% were found in mosquitoes from Israel [78]. Overall, the detection of WNV strains from two clades and the observed genetic nucleotide divergence of about 4% in 10 mosquitoes from one area at a single

time point demonstrate the circulation of a genetically diverse WNV population in the Zambezi region of Namibia.

We could further show that the mosquito specimen that was infected with M6848-NA-2020 took a blood-meal on a human host indicating human exposure to WNV. We could not identify any vertebrate DNA remaining from blood feeding in the other nine WNV-positive mosquitoes. Although *Cx. univittatus* is primarily ornithophilic, it has been shown to also feed on humans and was found in human dwellings [72]. Maintenance of WNV in its enzootic cycle in southern Africa involves ornithophilic mosquitoes and a wide range of southern African avian species [72]. Taken together, the high WNV detection rate and genetic diversity, as well as human host sources indicate the public health relevance of WNV in Namibia. However, there is a general lack of knowledge on the ecology and epidemiology of WNV in Africa including the study region of this paper. Other knowledge gaps include unknown distribution and incidence patterns of WNV in Africa, as well as on species involved in its natural amplification cycles. Future studies should focus on endemic transmission cycles and geographic distribution of WNV, as well as human infection rates with WNV.

To our knowledge, so far only two other clade 2b strains have been detected. One was found in a bird in South Africa in 1958 and the other in a bird in Cyprus in 1968 [69]. We discovered an 18-nucleotide deletion in the 3' UTR of strain M6646-NA-2020 that was not present in the other strains of clade 2b. The 3'UTR region of WNV is known to harbor a variety of sequence elements that play a role in maintenance of correct viral RNA secondary structures, interaction with cellular proteins and protecting the viral genome from degradation by RNases [79, 80]. These highly conserved, functional regions are located in the distal part of flavivirus UTRs, close to the genome 3'-end. However, the downstream part of the flaviviral 3' UTR that closely follows the 3'-end of the CDS, is well known for its high variability and typically contains insertions and deletions [81].

Unfortunately, we were not successful in isolating any of the WNV strains in cell culture although genome copies of up to 6.8 x 10^7 per mosquito were found. This is most probably due to thawing of the mosquito samples during their transport to the laboratory in Germany as the dryshipper was damaged on the flight. In contrast, all detected BAGV strains were successfully isolated in cell culture from mosquitoes. These samples were transported previously, and no damage was observed.

Three BAGV strains were uncovered in *Cx. univittatus* mosquitoes from Mudumu National Park in the Zambezi region. BAGV has previously been isolated from several species of the *Culex* genus [32, 34, 35] including *Cx. univittatus* [33]. BAGV is maintained in nature by transmission between wild birds of the Phasianidae family and *Culex* spp. [82]. Susceptible species also include farmed game birds like turkeys and wild game birds like pheasants and partridges [26-28, 31]. Several members of the Phasianidae family, like *Peliperdix coqui*, *Scleroptila gutturalis*, *Dendroperdix sephaena*, *Pternistis adspersus*, and *Pternistis swainsonii* are endemic in north-eastern Namibia [83]. However, little is known on BAGV maintenance in the Zambezi region. Screening of potential natural bird reservoir species for antibodies against BAGV could provide further insight into endemic transmission cycles, geographic distribution, and virus prevalence. In this study, growth analyses revealed great differences between cells derived from hosts involved in the natural virus transmission cycle, mosquito and bird cell lines, and mammalian cell lines derived from primates, rodents, cattle, and humans. Viral genome copy numbers were on average 100,000 times higher in mosquito and bird cells than in mammalian cells, except for cattle cells which were not susceptible. The mammalian cell lines BHK-21 and VeroE6 have been reported to support BAGV replication [30]. We found that the human cell line HEK293T enabled BAGV replication to a similar extent than BHK-21 and VeroE6 cells. Possibly BAGV is capable of infecting humans as neutralizing antibodies were found in encephalitic patients from India [36]. Such susceptibility of human cells for BAGV infection would indicate that humans may not be refractory for BAGV infection. We further found that muscovy duck cells (*Cairina moschata*, Anatidae) support BAGV replication to similar levels as mosquito cells, whereas chicken-derived cells were not susceptible. Although muscovy ducks are endemic to Central America, these findings may imply that BAGV may infect a wider range of bird species than partridges and pheasants. In Spain common wood pigeons (*Columba palumbus*, Columbidae) can also be infected by BAGV [84]. Thus, testing a wide variety of different bird species of the Zambezi region will help to identify the susceptible species and provide insight into virus maintenance in nature.

The high detection rate of insect-specific flaviviruses (95%, 241 of 254 pools positive) in the sample set confirmed findings from other studies, providing further evidence that ISFs are widely spread in mosquito populations worldwide, now including southern Africa [85-88]. Co-infection studies have shown that infection with insect-specific

viruses can alter replication and transmission of arboviruses in mosquitoes [89]. Notably, an inhibition of WNV replication mediated by a variety of ISFs in mosquitoes has been reported [90-93]. In contrast, Culex flavivirus, that was also found in mosquitoes of this study (**Fig 3**), seems to be positively associated with WNV infections in mosquitoes [94]. We have not observed any co-infections with multiple flaviviruses in our samples. However, *in vitro* co-infection experiments with arboviruses and ISFs detected in this study could identify if these viruses could have an effect on vector competence or transmission rates.

In conclusion, we demonstrated that a wide genetic diversity of flaviviruses is circulating in mosquitoes in the Zambezi region of north-eastern Namibia. Importantly, we identified two clusters of WNV belonging to two different clades and BAGV. WNV and BAGV can cause outbreaks, including severe disease and mortality in humans and birds, respectively. Further studies are needed to assess their health impact, as well as their social and economic impact in southern Africa.

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Supporting information

Table S1. Primers used for BAGV genome amplification.

Primer name	Primer sequence (5’ – 3’)
BAGV F1	TGACAGCTCAACACAAGTGC
BAGV R1	CTGGTTGACTGTGACAAGTGG
BAGV R1n	CCTGAGCCACCCCTAACAC
BAGV F2	AGTAGGGCCAGTCAGAATCG

BAGV F2n	CTTTGAAACCAACGGCAGGA
BAGV R2	ATGAGCCAGCAGATGATCCA
BAGV R2n	TGTTCACAAGCCAACTCTTCG
BAGV F3	CCGAAAGCACCAGTGTACAC
BAGV F3n	CTCTGGGATGGACATGAGCC
BAGV R3	TGCTTTGTCGCCCTTAATGG
BAGV R3n	CCTTAGTCCTAACCAAACTTTCG
BAGV F4	TCACTCCACCCATGTCCAAT
BAGV F4n	GCCCAGATGAGAATAGAGCCT
BAGV R4	CCCATCACAATCTATCTCAACGT
BAGV R4n	CTCCAGCCACAAGTCAGTCG
BAGV F5	AGTGAGTGAAGCCCTTACGG
BAGV F5n	GGGATCGCACACTTTGACG
BAGV R5	TGCATTTCCGACTGTCAATGA
BAGV R5n	CAAAGTCCCATTCGCCTGTT
BAGV F6	AAGACAGTGTGGTTTGTTCCT
BAGV F6n	GTGTGTCTGACGAAGGCC
BAGV R6	TTGCCACGATTCCATCAACC
BAGV R6n	CCAGCCGGGGATCAGAAA
BAGV F7	TTGGTTTGGGAAAGGGCATG
BAGV F7n	ACTGTTAGGGTGTTGGGGTC
BAGV R7	TGAACTCCTCCTTTGTGCAGA
BAGV R7n	CATCCATCGGGCTGTGATTT
BAGV F8	AAACCTTGGGACGCCTTACA
BAGV F8n	GACCGACACAACCCCATTTG
BAGV R8	CGGTTCAGAGTTGGCATGTAG
BAGV R8n	GGGCTCTGATCTGCATAATTGG
BAGV F9	GGGTGTGGATTGAGGAGAATG
BAGV F9n	CACATGGAAGACAAGACCCC
BAGV R9	GTGTTCTACCACCACCAGC

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Chapter II: Orbiviruses in biting midges and mosquitoes from the Zambezi region, Namibia

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Abstract

The genus *Orbivirus* includes a variety of pathogenic viruses that are transmitted by biting midges, mosquitoes, and ticks. Some of the economically most relevant orbiviruses are endemic to Namibia, like the livestock-pathogenic Bluetongue and African horse sickness viruses. Here, we assessed the diversity of orbiviruses circulating in the Zambezi region of north-eastern Namibia. A total of 10,250 biting midges and 10,206 mosquitoes were collected and screened for orbivirus infections. We identified Palyam virus (PALV) in a pool of biting midges (*Culicoides* sp.) sampled in the Wuparo Conservancy and three strains of Corriparta virus (CORV) in *Culex sp.* mosquitoes sampled in Mudumu National Park and the Mashi Conservancy. This is, to our knowledge, the first detection of PALV and CORV in Namibia. Both viruses infect vertebrates but only PALV has been reported to cause disease. PALV can cause foetal malformations and abortions in ruminants. Further, a novel orbivirus, related to Kammavanpettai virus from India and Umatilla virus from North America, was discovered in biting midges (*Culicoides* sp.) originating from Mudumu National Park and tentatively named Mudumu virus (MUMUV). Complete genomes of PALV, CORV and MUMUV were sequenced and genetically characterized. The Namibian CORV strain showed 24,3% nucleotide divergence in its subcore shell gene to CORV strains from Australia indicating that African CORV variants vary widely from their Australian relatives. CORV was isolated in cell culture and replicated to high titres in mosquito

and duck cells. No growth was found in rodent and primate cells. The data presented here show that diverse orbiviruses are endemic to the Zambesi region. Further studies are needed to assess their effects on wildlife and livestock.

Introduction

Orbiviruses infect a broad variety of vertebrates, including humans, cattle, horses, birds, rodents and bats, and are transmitted by blood-feeding arthropods, such as mosquitoes, sandflies, ticks and biting midges (of the genus *Culicoides*) [1]. The medically and economically most important orbiviruses are Bluetongue virus (BTV), African horse sickness virus (AHSV) and epizootic haemorrhagic disease virus (EHDV), which are all transmitted by biting midges and can cause high mortality outbreaks in livestock [2-4].

The genus *Orbivirus* (subfamily *Sedoreovirinae*) has 22 species that are recognised by the International Committee for the Taxonomy of Viruses (ICTV) and several unclassified members; it is the largest genus in the family *Reoviridae* [5]. The genome of orbiviruses consists of ten segments of double stranded RNA (dsRNA), coding for seven structural (VP1–VP7) and four non-structural (NS1–NS4) proteins [1]. Orbiviruses form nonenveloped virions of icosahedral shape with a diameter of approximately 90 nm. The virion consists of three shell layers, the outer capsid, the core-surface layer (an intermediate capsid) and the subcore shell (an inner capsid). Sequence divergence of RNA-directed RNA polymerase (RdRp) or the subcore shell genes are used for sequence-based taxonomic classification of members of a species or designation of new species, respectively [6].

Palyam virus (PALV) is transmitted by mosquitoes and biting midges and infections can cause foetal malformations and abortions in ruminants [7-9]. PALV shows a wide geographic distribution and has been found in Africa, Asia and Australia [10]. It was first isolated from *Culex vishnui* mosquitoes in India in 1956 [11]. So far, 13 distinct PALV serotypes have been identified that largely group according to their geographic origin in phylogenetic analyses [12].

Corriparta virus (CORV) is another orbivirus with a wide geographic distribution and host range. CORV has been found in Australia, Ethiopia, the Central African Republic, Brazil, and the USA [13, 14]. It was detected in mosquitoes and birds [15, 16], and neutralizing antibodies were found in humans [17, 18], cattle [18], horses [18], and

birds [15], but no disease is associated with a CORV infection. So far only two CORV genomes derived from Australian mosquitoes are available [19, 20]. All other detections relied on serological characterization, RNA–RNA blot hybridization, or sequencing of short genome fragments of less than 140 nucleotides [13, 21].

Kammavanpettai virus (KVPTV) was isolated from a wild bird in India in 1963 [22] but only recently described [23]. KVPTV is closely related to Umatilla virus and Stretch Lagoon orbivirus and probably represents a novel species. Umatilla virus was isolated from a wild bird and *Culex pipiens* mosquitoes in the USA [24], and Stretch Lagoon orbivirus was found in *Culex annulirostris* mosquitoes in Australia [25]. Neutralizing antibodies against Stretch Lagoon orbivirus have been found in horses, donkeys, and goats [26].

Several orbiviruses have been found in southern Africa. Serological data indicated that BTV, AHSV and EHDV are endemic to Namibia, on the basis of high seroprevalence rates in ruminants and ungulates [27-30]. PALV is widespread in South Africa and Zimbabwe but has so far not been detected in other countries in southern Africa [8, 31]. Antibodies against equine encephalosis virus (EEV) and Chenuda virus have been found in domestic and wild animals in Namibia and other countries in the region [32-35]. However, vector populations have not been monitored for infections with arboviruses for more than 35 years in Namibia. Here, we collected biting midges and mosquitoes in the Zambezi region in Namibia and tested them for infection with orbiviruses.

Methods

Mosquito and biting midge sampling

Mosquitoes and biting midges were sampled in north-eastern Namibia in the Sachinga Livestock Development Centre in May and June 2018, in Mudumu National Park and Wuparo Conservancy in November and December 2018, in Bwabwata National Park in February and March 2019 and in Mashi Conservancy in March and April 2019 and in February and March 2020 as described [36]. In the Sachinga Livestock Development Centre three sites and in all other locations four different sites were selected. CDC Light and Gravid traps (John W. Hock Company, Gainesville, United States of America) and BG Sentinel traps (Biogents AG, Regensburg, Germany) were operated from dusk till dawn for five consecutive days at each site (total trapping effort 1,480 trap nights).

BG Sentinel traps were either baited with BG-Lure (Biogents AG, Regensburg, Germany), 1-Octen-3-ol or worn socks. Samples were stored in liquid nitrogen in the field. Mosquito species were identified morphologically in the laboratory using the standard keys [37-39]. Biting midges were not identified morphologically, but only separated from additional bycatch.

Viral RNA extraction and PCR screening

Individual mosquito specimens were homogenized in 500 µl phosphate-buffered saline (PBS) using ceramic beads and a Qiagen Tissue Lyser (QIAGEN), and subsequently arranged into pools of 10 according to species and sampling location. Biting midges were sorted into 410 pools of 25 specimens each and homogenized in 500 µl PBS. Of these, 108 pools represented samples collected in Mudumu National Park and 302 pools were collected in the Wuparo Conservancy. RNA was extracted from 200 µl of the insect homogenates using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics, Mannheim, Germany). RNA was eluted in 100 µl of provided elution buffer. Subsequent cDNA synthesis was performed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers (Integrated DNA Technologies). All pools were screened for orbiviruses by generic RT-PCR targeting the RdRp gene located on the viral segment S1 using the primers Orbi-F1 5'-TTCTGGATWTGGiGARGG-3', Orbi-F2 5'-TGGAACATTRTGGARYGG-3', Orbi-R1 5'-AACGTATCRTCiCCMAC-3', Orbi-R2 5'-GTCGAGTTYTCRCCiGA-3', and Orbi-R3 5'-TGCAAGTGTiGARTTYTC-3'. Nucleotide sequences were characterized by Sanger sequencing (Microsynth Seqlab), analysed in Geneious 9.1.8 (Biomatters) and compared to GenBank by using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genome sequencing and analysis

RNA was extracted using QIAzol (QIAGEN) and Next generation sequencing was performed using the MiSeq Reagent kit v3 and the MiSeq desktop sequencer (Illumina). Paired end reads were assembled in Geneious 9.1.8 (Biomatters). The contigs were mapped using alignments of orbiviral sequences available from GenBank. Sequence gaps were closed by conventional PCR and subsequent Sanger sequencing (Microsynth Seqlab, Göttingen, Germany). The genomic ends of CP67-NA-2018 segment (S) 1 and the 5' termini of the segments S1, S4, and S8 and the 3'

termini of S6 of MP416-NA-2018 were obtained using the 5'-RACE System (Thermo Fisher Scientific). The genomic ends of CP279-NA-2018 segments S2, S5, S6, and S10 were obtained using generic primers binding to the 3'- and 5' segment termini based on an alignment of all available PALV sequences. Putative ORFs were identified by Geneious and transduced protein sequences were compared to GenBank using BLASTp.

Phylogenetic analysis

Nucleotide and amino acid sequences of viral open reading frames (ORFs) were aligned with related viral sequences in Geneious using MAFFT [40]. The VP1 (RdRP) phylogeny based on different orbivirus species was inferred using the Smart Model Selection (SMS) in PhyML [41]. All other phylogenies were inferred using PhyML [42] with the best models selected by mega X [43]. The phylogenetic tree based on the VP3 (T2) amino acid sequences was computed with the LG model using a fixed Gamma shape parameter of 1.38 and an estimated fraction of invariable sites. The phylogenetic tree based on the VP7 (T13) amino acid sequences was inferred with the Dayhoff model using an estimated fraction of invariable sites and an estimated Gamma shape parameter. The PALV phylogeny based on viral VP1 (RdRP) nucleotide sequences was computed using the GTR model with an estimated fraction of invariable sites and an estimated Gamma shape parameter. The phylogenetic tree of PALV VP3 (T2) nucleotide sequences was inferred with the HKY85 model using an estimated fraction of invariable sites and an estimated Gamma shape parameter. The phylogenetic relationship based on the PALV VP7 (T13) nucleotide sequences was computed with the HKY85 model using a fixed Gamma shape parameter of 29.57 and a fraction of 0.73 invariable sites. Confidence testing was performed based on 1,000 bootstrap iterations.

Virus isolation in cell culture

For virus isolation attempts in cell culture, cells derived from biting midges (KC, *Culicoides variipennis*), mosquitoes (C6/36, *Aedes albopictus*) and primates (VeroE6, African green monkey kidney) were either inoculated with 50 µl of unfiltrated insect homogenate supplemented with 1% penicillin-streptomycin (Thermo Fisher Scientific) or with 50 µl of filtrated homogenate. The cultures were checked daily for cytopathic effects (CPE). An aliquot of the supernatant was used for infection of fresh cells 8 days

post-infection (dpi). This procedure was repeated four times. Cell culture supernatants of each passage were tested for viral replication by quantitative (q) PCR. A virus stock was prepared from infectious cell culture supernatant of MP416-NA-2018 passage 3. The number of infectious particles was determined by Tissue Culture Infectious Dose 50 (TCID50) end-point dilution assay [44, 45].

Virus growth kinetics

Different vertebrate (AGE1.CR, duck [46]; VeroE6, African green monkey; BHK-21, hamster; KN-R, cattle; HEK293, human) and insect (C6/36, *Aedes albopictus*) cell lines were infected in duplicates at a multiplicity of infection (MOI) of 0.1 and 0.01, respectively. Aliquots of cell culture supernatants were sampled every 24 h for 5 days and virus copy numbers were determined by qPCR using a plasmid-based standard dilution series.

Results

Detection of orbiviruses in mosquitoes and biting midges

In total, 10,206 mosquitoes were sampled between May 2018 and March 2020 in Mudumu and Bwabwata National Parks, in Wuparo and Mashi Conservancies, and in the Sachinga Livestock Development Center (LDC) of Namibia's northeastern Zambezi region. Further, an estimated several thousand of biting midges were collected in Mudumu National Park and Wuparo Conservancy from November to December 2018. Mosquitoes were identified morphologically and sorted into 1,021 pools. In total, 41 species of mosquitoes were found (Table 1). A total of 1663 mosquitoes could only be identified to genus level and for 178 specimen no genus could be determined due to bad sample condition. A subset of 10,250 biting midges were sorted into 410 pools.

Table 1. Number of mosquitoes collected in the Zambezi region

Mosquito species	Proportion [n, %]
Aedeomyia Lepiothauma africana	96, 0.94%
Aedes Aedimorphus argenteopunctatus	3, 0.03%
Aedes Aedimorphus hirsutus	139, 1.36%
Aedes Aedimorphus ochraceus	1, 0.01%

Aedes Mucidus scatophagoides	1, 0.01%
Aedes Neomelaniconion circumluteolus	266, 2.61%
Aedes Pseudarmigeres argenteoventralis	1, 0.01%
Aedes Stegomyia aegypti	11, 0.11%
Aedes Stegomyia chaussieri	19, 0.19%
Aedes Stegomyia luteocephalus	1, 0.01%
Aedes Stegomyia metallicus	2, 0.02%
Aedes Stegomyia simpsoni	2, 0.02%
Aedes Stegomyia unilineatus	1, 0.01%
Aedes Zavortinkius fulgens	16, 0.16%
Anopheles Anopheles tenebrosus	1, 0.01%
Anopheles Cellia argenteolobatus	2, 0.02%
Anopheles Cellia buxtoni	1, 0.01%
Anopheles Cellia rufipes	11, 0.11%
Anopheles Cellia schwetzi	1, 0.01%
Anopheles Cellia squamosus	67, 0.66%
Coquillettidia Coquillettidia chrysosoma	7, 0.07%
Coquillettidia Coquillettidia cristata	1, 0.01%
Coquillettidia Coquillettidia fuscopennata	357, 3.50%
Coquillettidia Coquillettidia metallica	1851, 18.14%
Culex Culex antennatus	222, 2.18%
Culex Culex argenteopunctatus ssp. kingi	18, 0.18%
Culex Culex calurus	1, 0.01%
Culex Culex pipiens	96, 0.94%
Culex Culex quinquefasciatus	35, 0.34%
Culex Culex simpsoni	184, 1.80%
Culex Culex univittatus	3032, 29.71%
Culex Culiciomyia nebulosus Var. pseudocinereus	20, 0.20%
Culex culiciomyia semibrunneus	1, 0.01%
Culex Eumelanomyia rima	15, 0.15%
Culex Oculeomyia bitaeniorynchus	1, 0.01%
Culex Oculeomyia poicilipes	18, 0.18%

Ficalbia Etorleptiomyia mediolineata	16, 0.16%
Lutzia Metalutzia tigripes	8, 0.08%
Mansonia Mansonioides africana Var. nigerrima	185, 1.81%
Mansonia Mansonioides uniformis	1652, 16.19%
Uranotaenia Uranotaenia balfouri	3, 0.03%

The RT-PCR screening of all pools for infection with orbiviruses yielded five positive pools, i.e., the mosquito pools MP764-NA-2020 and MP799-NA-2020, both originating from Mashi Conservancy, and the mosquito pool MP416-NA-2018 originating from Mudumu National Park, as well as the pools consisting of biting midges CP67-NA-2018, sampled from Mudumu National Park and CP237-NA-2018, collected in Wuparo Conservancy (Table 2). Comparison of obtained sequence fragments with publicly available sequences in GenBank via the BLASTn algorithm revealed identities of 97% to PALV (CP279-NA-2018), 64% to CORV (MP416-NA-2018, MP764-NA-2020, and MP799-NA-2020), and 61% to KVPTV (CP67-NA-2018).

Table 2. Sampling information of detected orbiviruses

Virus	Strain	Host	Sampling location	Sampling date	Land-use type
Mudumu virus	CP67-NA-2018	*Culicoides sp.*	Mudumu National Park	11/19/2018	Undisturbed shrub savanna
Palyam virus	CP279-NA-2018	*Culicoides sp.*	Wuparo Conservancy	12/15/2018	Rangeland
Corriparta virus	MP416-NA-2018	*Culex sp.*	Mudumu National Park	11/20/2018	Undisturbed shrub savanna
	MP764-NA-2020	*Culex sp.*	Mashi Conservancy	03/15/2020	Agricultural field
	MP799-NA-2020	*Culex sp.*	Mashi Conservancy	03/15/2020	Agricultural field

Genome sequencing and genetic characterization

The genomes of MP416-NA-2018, CP67-NA-2018, and CP279-NA-2018 were sequenced by Next generation sequencing and representative segment termini of MP416-NA-2018, and CP67-NA-2018 were determined by RACE-PCR. According to the orbivirus nomenclature, segments were numbered according to size of encoded proteins although homologous proteins may consequently be encoded on different segments [47]. Genome organizations of CP279-NA-2018, MP416-NA-2018, and CP67-NA-2018 are summarized in Tables 3, 4, and 5, respectively.

Analysis of the entire genome of CP279-NA-2018 revealed the first detection of PALV in Namibia (Table 3). Maximum pairwise nucleotide (nt) and amino acid (aa) identities of the detected strain, named PALV CP279-NA-2018, were 95.2% and 98.5% for the RdRP, 96.9% and 99.6% for the sub-core shell T2 protein, and 96.7% and 99.0% for the major outer capsid protein between PALV CP279-NA-2018 and PALV strain Gweru VRL1726/76, respectively.

Table 3. Genome organization of PALV CP279-NA-2018

Segment	Putative encoded gene	ORF length (nt)	Putative function	Predicted protein length (aa)	GC content [%]	Accession no.
S1	VP1	3,888	RNA-dependent RNA polymerase	1,295	37.6	MW809629
S2	VP2	3,048	Outer capsid protein	1,015	38.8	MW809630
S3	VP3(T2)	2,712	Sub-core shell T2 protein	903	39.7	MW809631
S4	VP4	1,923	VP4 capping protein	640	39.9	MW809632
S5	NS1	1,638	Tubule-forming protein NS1	545	38.8	MW809633
S6	VP5	1,566	Outer capsid protein 2	521	41.4	MW809634

S7	VP7	1,047	Inner capsid protein VP7 (T13)	348	45.4	MW809635
S8	NS2	999	Viral inclusion body protein NS2	332	41.6	MW809636
S9	VP6	819	VP6 Helicase	272	41.4	MW809637
S9	NS4	252	non-structural protein NS4	83	49.6	MW809637
S10	NS3	636	NS3 protein	211	39.9	MW809638
S10	NS3a	282	NS3a protein	93	37.9	MW809638

The proteins encoded on the ten genome segments of MP416-NA-2018 showed maximum identity to the corresponding segments of CORV (Table 4). For example, the putative RdRP protein encoded on S1 showed 71.6% nt and 81.9% aa identities to the RdRP of CORV, and the protein encoded on S2 of MP416-NA-2018 showed 75.7% nt and 91.4% aa identities to the sub-core shell T2 protein of CORV, respectively. Similarly, the predicted major outer capsid protein encoded on S3 also showed maximum similarity to that of CORV (66.1%/68.7% nt/aa identities). According to the orbivirus species and genus demarcation criteria of the ICTV, members of the genus *Orbivirus* show more than 33% identity among their RdRP protein sequences [48], and members of one species share at least 76% identity in their sub-core shell T2 nucleotide sequences [48]. According to these criteria, the detected viruses MP416-NA-2018, MP764-NA-2020, and MP799-NA-2020 would thus be members of the classified virus species CORV, albeit with a borderline divergence value of 75.7%.

Table 4. Genome organization of CORV MP416-NA-2018

Segment	**Putative encoded gene**	**ORF length (nt)**	**Putative function**	**Predicted protein length (aa)**	**GC content [%]**	**Accession no.**
S1	VP1	3,864	RNA-dependent	1,287	41	MW809639

			RNA polymerase			
S2	VP3(T2)	2,874	Sub-core shell T2 protein	957	43.4	MW809640
S3	VP2	2,217	Outer capsid protein	738	41.3	MW809641
S4	VP4	1,935	VP4 core protein	644	44.9	MW809642
S5	NS1	1,770	Tubule-forming protein NS1	589	45.9	MW809643
S6	VP5	1,584	Outer capsid protein 2	527	46.5	MW809644
S7	NS2	1,098	Viral inclusion body protein NS2	365	47.6	MW809645
S8	VP7	1,065	Inner capsid protein VP7 (T13)	354	48.7	MW809646
S9	VP6	1,044	VP6 Helicase	347	47.1	MW809647
S9	NS4	486	non-structural protein NS4	161	51.4	MW809647
S10	NS3	717	NS3 protein	238	48.4	MW809648
S10	NS3a	249	NS3a protein	82	50.2	MW809648

Full genome analysis of CP67-NA-2018 revealed that the predicted RdRP protein encoded on S1 was most similar to the RdRP proteins of KVPTV (66.4%/64.8% nt/aa identities) and Umatilla virus (68.7%/62.2% nt/aa identities) (Table 5). The protein encoded on segment S2 of CP67-NA-2018 is most similar to the sub-core shell T2 protein of KVPTV (65.5%/62.0% nt/aa identities) and CORV (66.9%/50.1% nt/aa identities). The transduced protein of segment S3, showed a low but significant similarity to the amino acid sequence of the outer capsid proteins of KVPTV (135/462 aa, 29.2% identities) and CORV (66/280 aa, 23.6% identities). As CP67-NA-2018 shared less than 74% identity in its sub-core shell T2 nucleotide sequence with other

orbiviruses, the virus most likely belongs to a previously unknown virus species, tentatively named Mudumu virus (MUMUV) according to its place of origin.

Table 5. Genome organization of MUMUV CP67-NA-2018

Segment	Putative encoded gene	ORF length (nt)	Putative function	Predicted protein length (aa)	GC content [%]	Accession no.
S1	VP1	3,963	RNA-dependent RNA polymerase	1,320	40.1	MW815106
S2	VP3(T2)	2,907	Sub-core shell T2 protein	968	40.5	MW815107
S3	VP2	2,412	Outer capsid protein	803	38.6	MW815108
S4	VP4	1,956	VP4 core protein	651	40.4	MW815109
S5	NS1	1,776	Tubule-forming protein NS1	591	43.8	MW815110
S6	VP5	1,590	Outer capsid protein 2	529	39.4	MW815111
S7	NS2	1,305	Viral inclusion body protein	434	42.2	MW815112
S8	VP6	1,065	VP6 Helicase	354	42.7	MW815113
S8	NS4	480	non-structural protein NS4	159	47.7	MW815113
S9	VP7	1,056	Inner capsid protein VP7 (T13)	351	44.4	MW815114
S10	NS3	690	NS3 protein	229	41.2	MW815115
S10	NS3a	312	NS3a protein	103	38.5	MW815115

Phylogenetic relationship of detected orbiviruses

The results of maximum likelihood phylogenetic analyses based on VP1 (RdRP) (Fig. 1a), VP3 (T2) (Fig. 1b), and VP7 (T13) (Fig. 1c) proteins showed that PALV CP279-NA-2018 grouped with PALV, CORV MP416-NA-2018 grouped with CORV and Parry's Lagoon virus, an additional putative member of the CORV group [20], and MUMUV was placed as a sister taxon to KVPTV, forming a cluster with Strech Lagoon orbivirus and Umatilla virus. Of note, MUMUV was detected in biting midges but was placed within the mainly mosquito associated clade of orbiviurses [14]. This is in line with the presence of amino acids in the third conserved region of the RdRP that are usually mosquito and tick associated. For example, CP279-NA-2018 contained in motif B a W at position 729 in contrast to a T present in biting midges, in motif C a M at position 779 in contrast to a Q in biting midges, in motif D an A at position 829 in contrast to a V in biting midges, and in motif G a Y at position 390 whereas biting midges contained a F or L [23]. PALV 279-NA-2018, which was also detected in biting midges, grouped together with PALV, African horse sickness virus, Equine encephalosis virus, and Orungo virus within the group of mainly *Culicoides* associated orbiviruses [14].

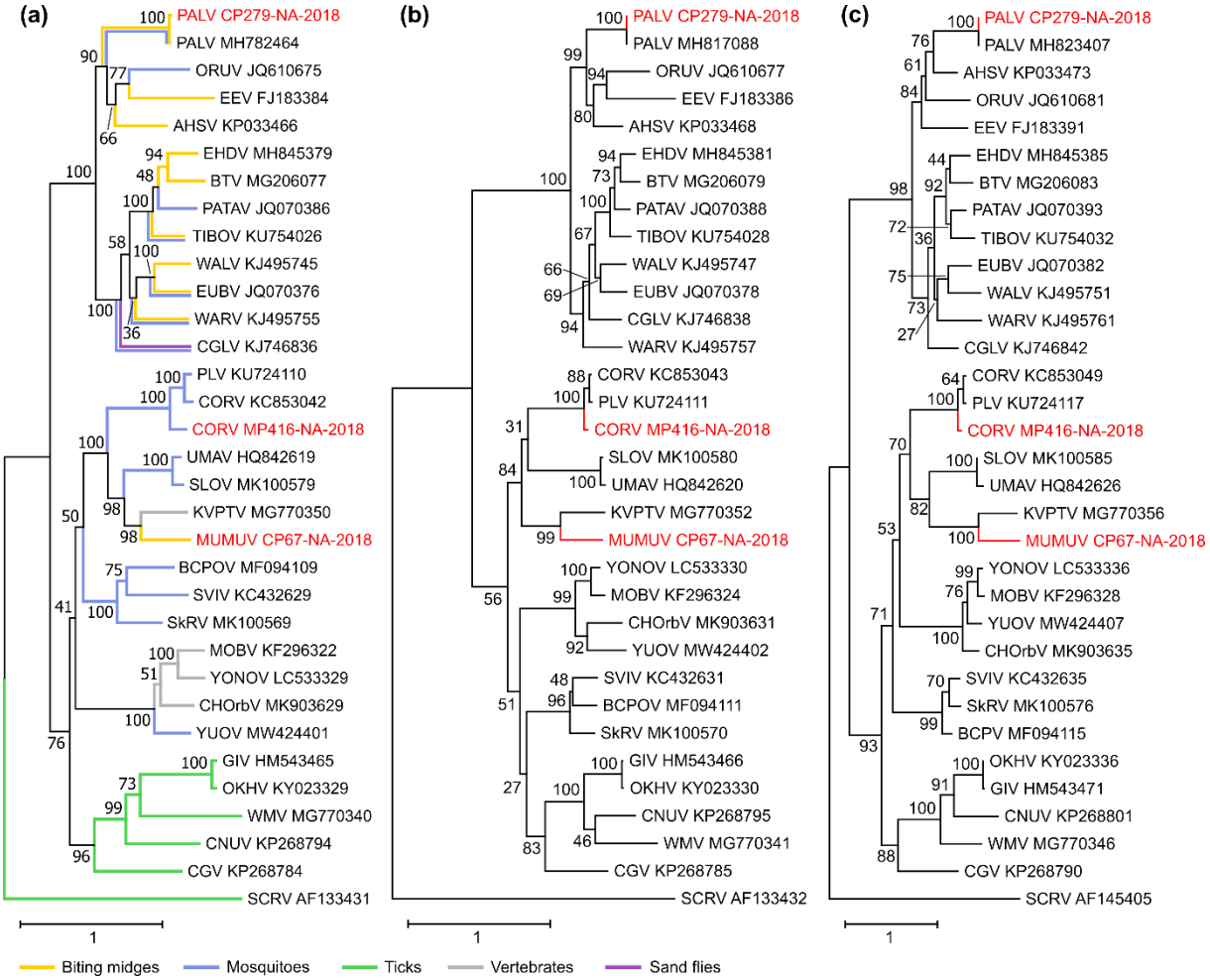

Fig. 1. Phylogenetic relationship of detected orbiviruses. Maximum-likelihood trees were based on the full amino acid sequences of the viral proteins VP1/RdRP (a), VP3/T2 (b), and VP7/T13 (c). Branch support was assessed by 1000 bootstrap replicates and is shown for each branch. Virus sequences detected in this work are shown in red. Accession numbers are given for reference sequences accessed from GenBank. Tree branches are marked in different colours according to host-associations. Virus name abbreviations are as follows: AHSV, African horse sickness virus; BCPOV, Big Cypress orbivirus; BTV, Bluetongue virus; CGLV, Changuinola virus; CGV, Chobar Gorge virus; CHOrbV, CHeRI orbivirus; CNUV, Chenuda virus; CORV, Corriparta virus; EEV, Equine encephalosis virus; EHDV, Epizootic haemorrhagic disease virus; EUBV, Eubenangee virus; GIV, Great Island virus; KVPTV, Kammavanpettai virus; MOBV, Mobuck virus; OKHV, Okhotsky virus; ORUV, Orungo virus; PALV, Palyam virus; PATAV, Pata virus; PLV, Parry's Lagoon virus; SCRV, St Croix River virus; SkRV, Skunk River virus; SLOV, Stretch Lagoon orbivirus; SVIV, Sathuvachari virus; TIBOV, Tibet orbivirus; UMAV, Umatilla virus; WALV, Wallal

virus; WARV, Warrego virus; WMV, Wad Medani virus; YONOV, Yonaguni orbivirus; YUOV, Yunnan orbivirus.

We further inferred additional phylogenies of PALV CP279-NA-2018 and all available PALV sequences for the viral VP1 (RdRP) (Fig. 2a), VP3 (T2) (Fig. 2b), and VP7 (T13) (Fig. 2c) genes. In all trees, PALV 279-NA-2018 grouped within the Afrotropical clade according to its geographic origin [12].

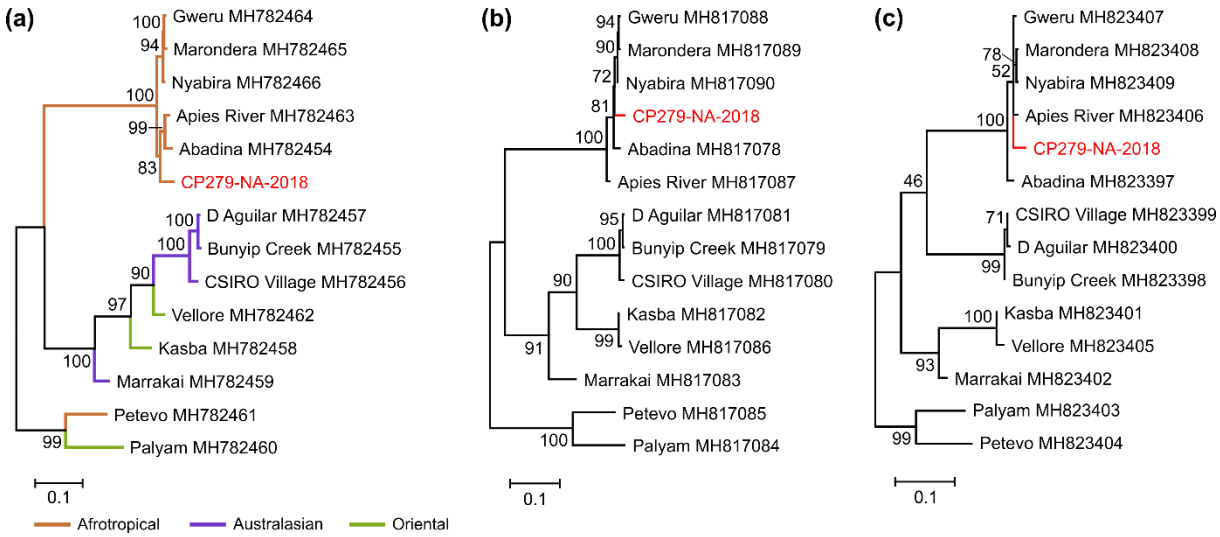

Fig. 2. Phylogenetic relationship of the Namibian PALV strain. Maximum likelihood trees are based on an alignment of entire coding sequences of the proteins VP1/RdRP (a), VP3/T2 (b), and VP7/T13 (c). Branch support was assessed by 1000 bootstrap replicates and is marked at each branch. The virus sequence from this study is shown in red. Reference sequences are marked with the serotype name and the respective GenBank accession number. Tree branches are marked in different colours, according to their geographic origin.

Growth analyses of CORV

PALV 279-NA-2018, MUMUV CP67-NA-2018 and CORV MP416-NA-2018 were inoculated into different insect and vertebrate cells. All attempts to isolate MUMUV CP67-NA-2018 and PALV 279-NA-2018 in cell culture failed, but isolation of CORV MP416-NA-2018 in C6/36 mosquito cells was successful.

Growth analyses in C6/36 cells showed the induction of a CPE four dpi and high genome copy numbers of almost 10^{11} copies ml^{-1} six dpi (Fig. 3). An array of different cell lines was infected with MP416-NA-2018 in order to assess virus growth and

putative host tropism. Virus growth of CORV MP416-NA-2018 in the avian cell line AGE1.CR reached medium levels of replication, with genome copy numbers approximately 10.000 fold below the level in C6/36 cells. MP416-NA-2018 did not replicate in BHK-21, HEK293T, and VeroE6 cells.

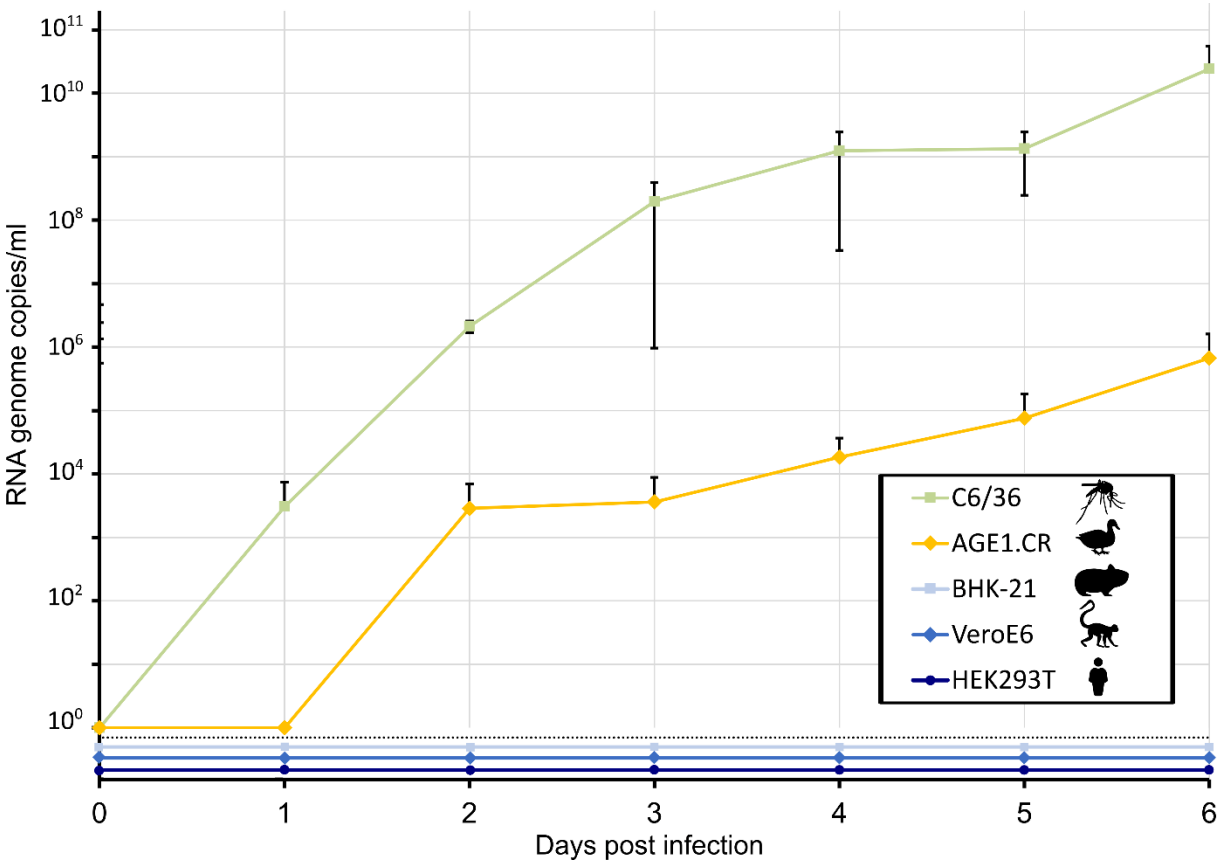

Fig. 3. CORV MP416-NA-2018 growth in vertebrate and insect cell lines. The mosquito cell line C6/36 (derived from *Aedes albopictus*) was infected in duplicates at an MOI of 0.01. Mammalian cell lines BHK-21 (hamster), HEK293T (human), VeroE6 (monkey), and the avian cell line AGE1.CR (duck) were infected in duplicates at an MOI of 0.1. A sample of cell culture supernatant was taken every 24 h for five consecutive days and viral copy numbers were determined by RT-qPCR.

Discussion

Orbiviruses are transmitted by blood-feeding arthropods and can cause severe diseases in animals and humans [1]. Screening of vector populations can provide early indications of circulating arboviruses. In this study we tested biting midges and mosquitoes from the Zambezi region of north-eastern Namibia for infections with

orbiviruses. We report the first identification, to our knowledge, of PALV and CORV in Namibia. PALV was detected in biting midges (PALV CP279-NA-2018) and CORV in mosquitoes (CORV MP416-NA-2018, MP764-NA-2020 and MP799-NA-2020). We further identified a previously unknown orbivirus, tentatively termed MUMUV, in biting midges (MUMUV CP67-NA-2018).

Most probably PALV has been circulating undetected in Namibia for a long time, as members of the PALV serogroup are widespread across Africa, including South Africa and Zimbabwe [8, 31, 49]. First reports of the detection of PALV in aborted cattle foetuses in Zimbabwe date back to 1976 [7], and a few years later PALV neutralizing antibodies were widely found in cattle sera in Zimbabwe [50]. Infections are mostly asymptomatic in adult cattle and goats but cause congenital malformation and abortion in pregnant livestock as well as severe disease in new-born animals, such as ataxia and neurological symptoms [7-9]. Of the 13 PALV serotypes described to date, especially the African strains Nyabira, Abadina, Gweru, Marondera, and Apies River and the oriental strain Kasba (Chuzan) are associated with pathogenicity in ruminants [8, 9, 31]. In contrast, the Australian PALV strains D'Aguilar, Bunyip Creek, CSIRO Village, and Marrakai, have not been implicated in diseases so far [51, 52]. The newly detected Namibian PALV strain CP279-NA-2018 forms a monophyletic clade with the other African serotypes in phylogenetic analyses (Fig. 2). It groups with Abadina and Apies River serotypes in VP1 based phylogenies that have been detected in Nigeria and South Africa, respectively [31, 53]. The close relationship between CP279-NA-2018 and pathogenic PALV serotypes suggests a similar pathogenic potential for the newly detected strain. Interestingly, CP279-NA-2018 was found in biting midges sampled at bovine-frequented rangeland areas in the Wuparo Conservancy, thus providing an opportunity of infecting cattle. Outbreaks affecting ruminants can have particularly dire consequences in the region, since many traditional southern African communities rely on cattle as a basis for their livelihoods, and the production of livestock has always been regarded a major part of rural development in the dry parts of the region [54-56]. Outbreaks can have the potential for devastating effects on peoples' livelihoods by causing significant loss of livestock and status, when met unprepared [57]. In summary, our data suggest that PALV is circulating the Zambezi region. However, further sero-epidemiological studies involving screening of cattle and other livestock sera for antibodies against PALV and acute infections are needed to assess if CP279-NA-2018 infects livestock and causes diseases in the region.

We further detected three strains of CORV in *Culex sp.* mosquitoes sampled in Mudumu National Park and Wuparo Conservancy and provide the full virus genome sequence for one of the strains, CORV MP416-NA-2018. CORV was first found in mosquitoes in Australia in 1960 [16], and serologically related viruses have been found subsequently in Africa and South America. However, no sequence data from previous CORV detections in Ethiopia and the Central African Republic are available [13]. Hence, we provide here the first CORV sequence from the African continent. Sequence divergence of the sub-core shell T2 gene is used as species demarcation criteria, with a threshold of at least 76% nucleotide identity among members of the same species [58]. CORV MP416-NA-2018 shows 75.7% pairwise identity to the sub-core shell T2 nucleotide sequence of the original CORV strain from Australia [16, 19]. The detected virus, thus, represents a genetically distant member of the CORV species. Growth analyses of CORV MP416-NA-2018 in cell culture showed no replication in BHK-21 cells, as has been repeatedly reported for the original Australian strain CORV MRM1 [15, 19, 59]. Medium virus permissiveness of the avian AGE1.CR cell line suggests birds as potential hosts, as implicated in previous studies by the detection of neutralizing antibodies against CORV and the isolation of the virus from a wild bird [15, 60]. Serological data from Australia suggest that CORV MRM1 infects cattle and humans, but so far, no signs of disease have been observed [17, 18]. Recently a closely related virus, termed Parry's Lagoon virus, was discovered in Australia that seems to be unable to infect vertebrate cells and might be restricted to insects as hosts [20]. Sero-epidemiological studies, similar to the ones previously outlined for PALV, are needed to identify the host range of CORV MP416-NA-2018 and potentially associated signs of disease.

Finally, we also discovered a proposed new member of the genus *Orbivirus*, tentatively named Mudumu virus (MUMUV) after its place of origin. According to ICTV standards, distinct virus species within the genus *Orbivirus* share at most 76% sub-core shell T2 nucleotide sequence identity with their next relative [58]. MUMUV CP67-NA-2018 was closest related to KVPTV. In pairwise sequence alignments, the putative sub-core shell T2 genes of the two viruses only showed 59.1% identity. This clearly falls below the cut-off value of 76%, suggesting the discovery of a genetically distinct new virus species. Interestingly, in the phylogenetic tree in Fig. 1, MUMUV CP67-NA-2018 grouped with the mainly mosquito-associated viruses, despite being isolated from biting midges. Furthermore, the subcore-shell protein T2 and the major outer capsid

protein CP67-NA-2018 are encoded on S2 and S3, respectively, which is typical for mosquito- and tick-associated orbiviruses [47]. *Culicoides*-borne orbiviruses usually encode the subcore-shell protein T2 on S3. Nevertheless, there is no strict phylogenetic grouping of orbiviruses according to their hosts as several orbiviruses, like PALV, Warrego virus, Wongorr virus, Tibet orbivirus and Eubenangee virus have been found in Culicoides and mosquito hosts [12, 61-63]. MUMUV may thus also be vectored by mosquitoes. The closest relatives to MUMUV in the phylogeny are the putative arbovirus KVPTV and the arbovirus Umatilla virus, which have both been isolated from wild birds [22, 24]. KVPTV induces clinical symptoms and death, when injected into newborn CD1 mice [23] and Umatilla virus induces CPE in BHK-21 cells [24]. Furthermore, for the allied Stretch Lagoon orbivirus, neutralizing antibodies have been found in horses, donkeys and goats in Australia [26]. The distinct grouping and high genetic relatedness with putative mosquito-borne viruses assume the isolation of a novel arbovirus that may infect birds.

In summary, we demonstrated a high genetic diversity of orbiviruses endemic to the Zambezi region of north-eastern Namibia. Considering the high economic importance of arboviruses in the region, especially for the livestock industry, these findings call for more in-depth sero-epidemiological studies, increased surveillance and potentially launching vector control measures.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter III: Eilat alphavirus isolated from *Culex univittatus* mosquitoes from the Namibian Zambezi Region suppresses in vitro replication of flavi- and alphaviruses

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Abstract

The genus *Alphavirus* harbors arboviruses of great concern, such as the Chikungunya virus (CHIKV) and the equine encephalitis viruses. Transmission of pathogenic alphaviruses by mosquitoes could be impaired by insect-specific alphaviruses such as Eilat virus (EILV). However, insect-specific alphaviruses are rarely found in wild mosquitoes and only a few have been described in the literature. Here, we report the detection of EILV in a *Culex univittatus* mosquito from the north-eastern Namibian Zambezi Region. Full genome analysis of MP458-NA-2018 showed 94.5% nucleotide identity to an EILV isolate from Israel. MP458-NA-2018 grouped with EILV in phylogenetic analysis and was placed within the clade of insect-specific alphaviruses. The virus was isolated in mosquito cells and shown to be restricted to insects as hosts by the inability to infect different vertebrate cell lines and a block of virus replication at temperatures over 34°C. We further show that infection of cells with EILV MP458-NA-2018 reduced viral replication rates of CHIKV and Sindbis virus (SINV) by 7-500-fold. We also observed that EILV MP458-NA-2018 reduced the replication rates of two endemic flaviviruses, West Nile virus (WNV) and Bagaza virus (BAGV) by 5-10 fold. In summary, EILV from the Namibian Zambezi Region suppresses *in vitro* replication of endemic flavi- and alphaviruses.

Introduction

The genus *Alphavirus* comprises 32 species and is the only genus in the family *Togaviridae* [1]. Alphaviruses are divided into a diverse group infecting a wide range of vertebrates ranging from mammals to birds and fish that are transmitted by

arthropods [2] and a monophyletic less diverse group of viruses with a host range restricted to insects [3]. Alphaviruses are found on all continents [4]. The genus contains several important human and animal pathogenic viruses, like the Chikungunya virus (CHIKV), Sindbis virus (SINV) and eastern equine encephalitis virus (EEEV) [5].

Alphaviruses form enveloped virions of spherical shape with a diameter of around 65 to 70 nm [6]. Their genome is composed of single-stranded positive-sense RNA, approximately 11,000 to 12,000 nucleotides in length, with a 5' cap structure and 3' poly(A) tail, which is coding for four non-structural (NSP1-NSP4) and six structural proteins (SPs) (Capsid, E3, E2, 6K/TF, and E1) [6].

Eilat virus (EILV) was the first insect-specific alphavirus to be described, found in a pool of *Anopheles coustani*, sampled in the Negev desert of southern Israel in the early 1980s [7]. EILV grows to high viral titers in insect cells but neither infects mammalian cells nor infant mice [7]. Several barriers of host range restriction of EILV to insects were identified including inhibition of entry and attachment, as well as a restriction of genomic RNA replication [7-9]. The inability of EILV to replicate in mammalian cells has been used to construct a safe to handle CHIKV antigen and a CHIKV vaccine, based on EILV(NSPs)/CHIKV(SPs) chimeric virions [10, 11].

Several other alphaviruses also displaying a host range exclusively restricted to insects were found hereafter, such as the Taï Forest alphavirus (TALV) from Ivory Coast [12], Mwinilunga alphavirus (MWAV) from Zambia [13], Agua Salud alphavirus (ASALV) from Panama [14], and Yada Yada virus (YYV) from Australia [15].

EILV and other insect-specific viruses (ISVs) were shown to affect *in vitro* and *in vivo* a second infection with another virus [16-21]. An effect, where a preexisting virus infection impairs a secondary infection with the same (homologous interference) or with a similar (heterologous interference) virus is termed superinfection exclusion (SIE) [22, 23]. Prior infection of C7/10 (*Aedes albopictus*) cells with EILV induced both homologous interference in subsequent infections with EILV, as well as heterologous interference in secondary infections with SINV, CHIKV, EEEV, western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV) [18]. A similar effect was observed *in vivo* for *Aedes aegypti* mosquitoes that were intrathoracic injected with EILV and orally superinfected with CHIKV [18]. Effects of SIE have been shown at various stages of the viral life cycle, including replication, translation, attachment, and penetration [24-29]. It is suggested that SIE in insects

might be mediated by viral proteins, such as the alphaviral NSP2 protein, or by the competition for host cell factors, which are necessary in the replication process [29, 30].

So far five human- and animal-pathogenic alphaviruses have been detected in southern Africa, SINV, Middelburg virus (MIDV), CHIKV, Ndumu virus, and Semliki Forest virus [31]. SINV and MIDV seem to be widely distributed in the region [32, 33]. Seroprevalence studies, conducted in the Namibian Zambezi Region in the 1960s, indicated the circulation of CHIKV and SINV [34] (HI and N). However, only low seroprevalence rates against CHIKV and SINV were found in the local population in the 1980s [35, 36]. Little is known on the diversity and prevalence of alphaviruses in vector populations in southern Africa. In this study we screened mosquitoes from Namibia's Zambezi Region for alphavirus infection and studied the effect of a detected insect-specific alphavirus on the replication of endemic vertebrate-pathogenic alphaviruses.

Methods

Mosquito collection and identification

Mosquitoes were sampled in the north-eastern Namibian Zambezi Region. Sampling was conducted in the Sachinga Livestock Development Centre between May and June 2018, in the Wuparo Conservancy and Mudumu National Park between November and December 2018, in the Mashi Conservancy between March and April 2019 and between February and March 2020, and in the Bwabwata National Park between February and March 2019 [37]. Samples were stored in the field in liquid nitrogen and mosquito species were identified morphologically in the laboratory using standard literature [38-40].

Viral RNA extraction and PCR screening

Individual mosquitoes were homogenized in phosphate-buffered saline (PBS) using a Tissue Lyser (QIAGEN, Hilden, Germany) system with ceramic beads, and homogenates were arranged into pools of 10 specimens. RNA was extracted with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics, Mannheim, Germany) from pooled mosquito homogenate and subsequently cDNA was synthesized with the SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific

GmbH, Dreieich, Germany) using random hexamer primers (Integrated DNA Technologies Germany GmbH, Munich, Germany). Samples were screened for alphavirus infection with a generic PCR assay using a published protocol [12]. Nucleotide sequences, derived by Sanger sequencing (Microsynth Seqlab GmbH, Göttingen, Germany), were analysed in Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) and by using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the GenBank database.

Genome sequencing and analysis

For full genome sequencing, viral RNA was extracted with the QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and Next generation sequencing (NGS) was performed on a MiSeq desktop sequencer, using the MiSeq Reagent kit v3 (Illumina Inc., San Diego, USA). Assembly of paired end reads was performed in Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand). Resulting contigs were mapped to EILV sequences as reference available from GenBank. The virus genomic sequence was confirmed by conventional PCR and Sanger sequencing (Microsynth Seqlab GmbH, Göttingen, Germany). The genomic ends of EILV MP458-NA-2018 were obtained using the 5'-RACE kit (Thermo Fisher Scientific GmbH, Dreieich, Germany). Putative viral open reading frames (ORFs) were identified using Geneious. Transduced amino acid sequences were compared to GenBank using BLASTp.

Phylogenetic analysis

Nucleotide sequences of viral ORFs were translationally aligned with related alphaviral sequences in Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) using the MAFFT algorithm [41]. The alignment of SPs was based on the E2-6K-E1 polyprotein-ORF and the alignment based on NSP ORF, with NSP3 gene trimmed. All phylogenies were inferred with PhyML, using the Smart Model Selection (SMS) method [42, 43].

Mosquito species identification by genetic barcording

A cytochrome c oxidase I (COI) gene fragment was amplified with generic primers for invertebrates and primers designed for *Culex univittatus* mosquitoes (forward 5'-TTAGGAGCTCCAGACATAGCTTTC-3', reverse 5'-AGGTAATGATAAAAGTAATAAAACAGCAGT-3') using the alphavirus positive mosquito pool MP458-NA-2018 [44]. PCR products were sequenced by Sanger

sequencing (Microsynth Seqlab GmbH, Göttingen, Germany) and compared to the GenBank database using BLAST.

Virus isolation and plaque purification in cell culture

For virus isolation in cell culture, cells derived from mosquitoes (C6/36, *Aedes albopictus*) and primates (VeroE6, African green monkey kidney) were inoculated with the virus-positive mosquito homogenate MP458-NA-2018 and cultivated for four weeks as described earlier [45]. Cell culture supernatants of each passage were checked for virus growth by RT-PCR [12]. A virus stock was generated using the C6/36 cell culture supernatant of the first passage. A plaque purification was performed from the virus stock following established protocols [12, 14]. Another virus stock was prepared from the plaque purified virus and designated MP458-NA-2018-PP. The purity of the MP458-NA-2018-PP stock was confirmed by NGS. A Tissue Culture Infectious Dose 50 (TCID50) end-point dilution assay was used to determine the number of infectious particles for both virus stocks [46].

Virus growth kinetics

C6/36 cells were infected with MP458-NA-2018-PP in duplicates at a multiplicity of infection (MOI) of 0.1 and incubated at either 28°C, 31°C, or 34°C in order to evaluate the influence of different ambient temperatures on virus replication as described previously [47]. In addition, cells were infected with MP458-NA-2018 and incubated at 28°C. Aliquots of cell culture supernatant were taken every 24 hours for five consecutive days and viral genome copy numbers were assessed using qPCR with a plasmid-based standard dilution series as described earlier [37]. The qPCR assay was established based on a 184 nucleotide (nt) fragment of the viral RdRP gene (forward 5'-AACCAGCACACATCTACCCA-3', reverse 5'-TCCGTGTATGATGTTGTCGTC-3', probe 5'-FAM-CGGTTTGGTGCCATGATGAA-ZEN-3'). Further, cells derived from primates (VeroE6, African green monkey kidney), humans (HEK293T, human kidney), and rodents (BHK-21, hamster kidney) were inoculated with MP458-NA-2018-PP at a MOI of 1 and cultivated for four weeks. Cell culture supernatants were tested for viral replication by qPCR.

Superinfection experiments

The mosquito cell line C7/10, derived from *Aedes albopictus*, was infected with MP458-NA-2018-PP at an MOI of 1. The cells were additionally either infected with CHIKV, SINV, West Nile virus (WNV), or Bagaza virus (BAGV) at an MOI of 0.01 and 0.1 14 hours post infection with MP458-NA-2018-PP. Aliquots of cell culture supernatants were sampled every 24 hours for four consecutive days and CHIKV, WNV, and BAGV genome copy numbers were determined by qPCR as described [37, 48, 49]. SINV gene copy numbers were assessed with a newly established qPCR assay (forward 5'-TTGAATGTCGTTATCGCCAGC-3', reverse 5'-GTTGTCGTCGCCAATGAACG-3', probe 5'-FAM-AGCGGCTTAAAACGTCCAGA-ZEN-3').

Results

Screening for alphaviruses

RT-PCR screening of the 10,206 mosquitoes for alphavirus infection resulted in one positive pool of *Culex univittatus* mosquitoes, MP458-NA-2018, originating from Mudumu National Park. The sequence fragment showed maximum nucleotide identity of 95.5% to the original EILV isolate EO329 from Israel. The mosquito species was identified by morphology and confirmed by genetic barcoding.

Virus isolation and plaque purification

MP458-NA-2018 was successfully isolated in C6/36 cells. The virus induced a strong CPE in the first passage 3.5 dpi. As other insect-specific alphaviruses had been co-isolated together with negeviruses, the obtained isolate was plaque-purified to obtain a pure virus stock. The purified version of the virus (designated MP458-NA-2018-PP) induced small to medium sized plaques 3.5 dpi in C6/36 cells.

Genome sequencing and genetic characterization

The genome of MP458-NA-2018 and MP458-NA-2018-PP were sequenced by NGS and the genome termini were determined by RACE-PCR. Analysis of MP458-NA-2018-PP confirmed a pure virus stock, with 100% identity to the wild-type virus sequence. Analysis of the entire genome of MP458-NA-2018 confirmed the first detection of EILV in southern Africa. We observed a genome length of 11,706 nucleotides excluding the 3'-UTR poly(A) tail. MP458-NA-2018 which showed the

same alphavirus-typical genome organization as EILV isolate EO329. The NSP- and SP-ORFs of MP458-NA-2018 had pairwise nucleotide identities of 95.5% and 94.9% and pairwise amino acid identities of 97.8% and 97.7% to EILV EO329, respectively, indicating the detection of an EILV variant, tentatively designated EILV MP458-NA-2018. It included three conserved sequence elements (CSEs), which are identical to the ones published for EILV EO329, SP1 CSE (51-nt CSE), the subgenomic promoter CSE, and 3′ CSE. The 5’ CSE (44-nt CSE), however, differed from the sequence found in EILV EO329 in five nucleotide positions. The translated protein ORFs of EILV MP458-NA-2018 contained all protease cleavage sites, as well as the E1 fusion peptide and ribosomal binding site (RBS) motifs postulated for EILV EO329. The 5’-UTR of EILV MP458-NA-2018 was 57 nt in length and contained six nucleotide exchanges and a single nucleotide insertion when compared to EILV EO329. The 3’-UTR of MP458-NA-2018 was found to range between 619 to 625 nt caused by a poly(A) tail in the range from 11-mers to 17-mers. Interestingly, the 3’-UTR of MP458-NA-2018 showed an insertion of 89 nucleotides in its middle part, 25 nucleotide exchanges, and one deletion in comparison to the 3’-UTR of EILV EO329.

Phylogenetic analysis

Maximum likelihood phylogenetic analyses based on an alignment of the viral SPs showed that EILV MP458-NA-2018 groups with the EILV strains EO329 from Israel and “EILV-Morocco” (Figure 1A). As observed earlier, the EILV strains formed a distinct clade with the other insect-specific alphaviruses, MWAV, TALV, YYV, and ASALV, in sister relationship to viruses of the Western equine encephalitis (WEE) complex. EILV MP458-NA-2018 also grouped within the ISV alphaviruses in phylogenetic analyses based on the viral NSPs (Figure 1B), However, in this phylogeny the group of ISVs was placed as a sister clade to a clade formed by SINV, Whataroa virus, and Aura virus, with ASALV placed in basal position to both clades as reported previously in other studies [13-15].

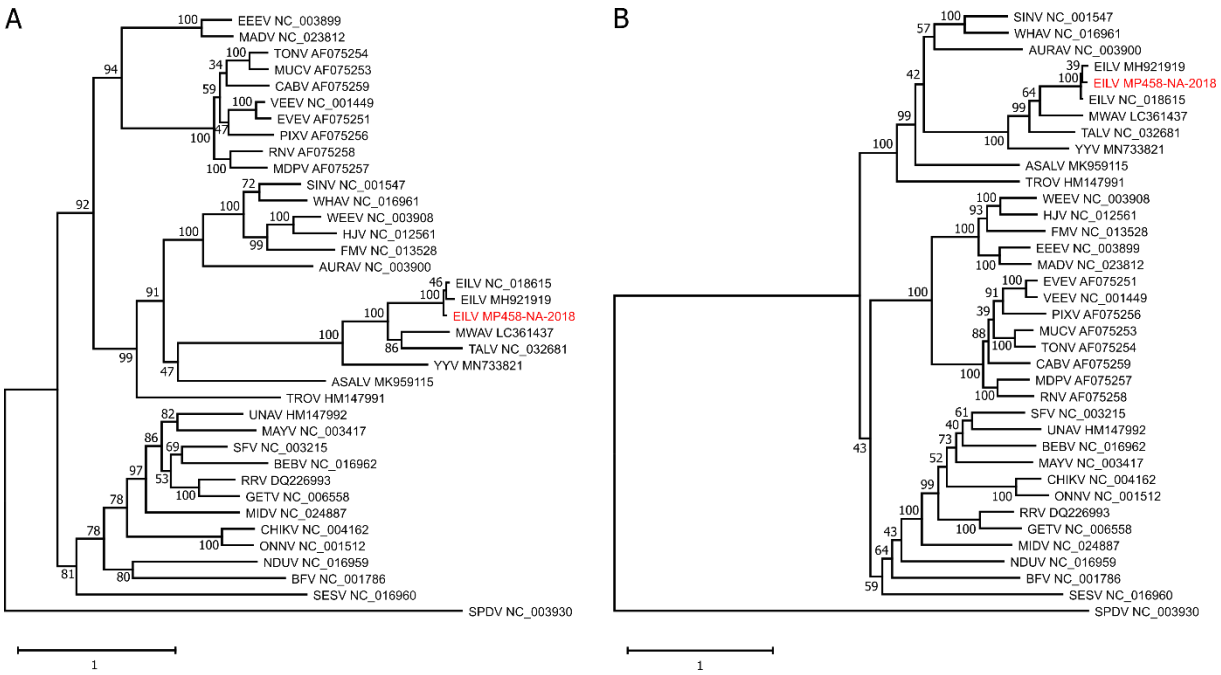

Figure 1: Phylogenetic relationship of the detected EILV strain MP458-NA-2018. Maximum likelihood trees were based on the nucleotide sequences of the viral SP ORF (A) and the viral NSP ORF (B). The phylogeny based on the viral SPs was calculated using the GTR model with an estimated proportion of invariable sites of 0.09 and estimated gamma shape parameter of 0.712, as chosen by Smart Model Selection in PhyML (SMS). The phylogenetic tree based on the viral NSPs was inferred with the GTR model with an estimated proportion of invariable sites of 0.167 and an estimated gamma shape parameter of 0.591. The phylogenetic trees were rooted on midpoint and confidence testing was performed based on 1,000 bootstrap iterations, as shown for each branch. Virus sequences detected in this work are shown in red. Accession numbers are given for reference sequences accessed from GenBank. Virus name abbreviations are as follows: ASALV, Agua Salud alphavirus; AURAV, Aura virus; BFV, Barmah Forest virus; BEBV, Bebaru virus; CABV, Cabassou virus; CHIKV, Chikungunya virus; EEEV, Eastern equine encephalitis virus; EILV, Eilat virus; EVEV, Everglades virus; FMV, Fort Morgan virus; GETV, Getah virus; HJV, Highlands J virus; MADV, Madariaga virus; MAYV, Mayaro virus; MIDV, Middelburg virus; MDPV, Mosso das Pedras virus; MUCV, Mucambo virus; MWAV, Mwinilunga alphavirus; NDUV, Ndumu virus; ONNV, O'nyong-nyong virus; PIXV, Pixuna virus; RNV, Rio Negro virus; RRV, Ross River virus; SPDV, Salmon pancreas disease virus; SFV, Semliki Forest virus; SINV, Sindbis virus; SESV, Southern elephant seal virus; TALV, Taï Forest alphavirus; TONV, Tonate virus; TROV, Trocara virus; UNAV, Una virus; VEEV,

Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WHAV, Whataroa virus; YYV, Yada Yada virus.

In vitro temperature sensibility and host range assessment

To assess if the Namibian EILV variant also shows a host range restriction to insects, MP458-NA-2018-PP was inoculated into different vertebrate and insect cells and cultivated for four weeks. Virus replication was detected by RT-qPCR. EILV MP458-NA-2018-PP did not replicate in any of the tested vertebrate cell lines, BHK-21, HEK293T, and VeroE6. Growth analyses in C6/36 cells showed high genome copy numbers and revealed no fitness impairment of the plaque purified virus (MP458-NA-2018-PP) in comparison to the wildtype (Figure 2A). To further test if MP458-NA-2018 cannot replicate at vertebrate body temperatures, C6/36 cells were infected with MP458-NA-2018-PP and incubated at different temperatures. Incubation at 31°C slightly impaired virus replication during the first 62 hours post infection (hpi) (Figure 2B). Similar gene copy numbers of 10^{11} copies/ml were reached at 3 dpi as well as when incubated at 28°C. No virus replication was detected at 34°C.

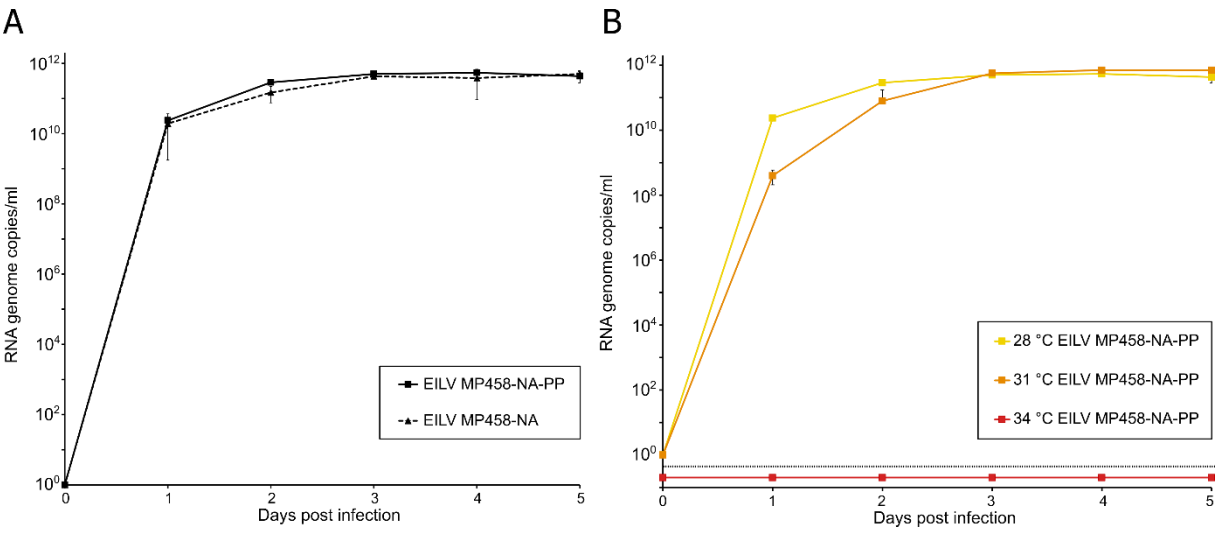

Figure 2: *In vitro* growth analysis and temperature sensitivity of EILV MP458-NA-2018. (**A**) Growth analysis of original and plaque-purified version of EILV MP458-NA-2018. The mosquito cell line C6/36 (derived from *Aedes albopictus*) was infected in duplicates at an MOI of 0.1. A sample of cell culture supernatant was taken every 24 hours for five consecutive days and viral copy numbers were determined by RT-qPCR. (**B**) Temperature sensitivity analysis of EILV MP458-NA-2018-PP. The mosquito cell line C6/36 (derived from *Aedes albopictus*) was infected in duplicates at an MOI of 0.1.

Cells were incubated at 28°C, 31°C, or 34°C. A sample of cell culture supernatant was taken every 24 hours for five consecutive days and viral copy numbers were determined by RT-qPCR.

Superinfection experiments

To test if the presence of EILV MP458-NA-2018 PP has an effect on superinfecting arboviruses, we selected the two alphaviruses CHIKV and SINV that are endemic in southern Africa and have been reported to be impaired by EILV [18], as well as the two flaviviruses WNV and BAGV that have been detected previously in the same region and same mosquito species [37] for superinfection experiments. C7/10 cells were either mock-infected or pre-infected with EILV MP458-NA-2018-PP and 16 hpi superinfected with CHIKV at an MOI of 0.01 and with either SINV, WNV or BAGV at an MOI of 0.1 as preliminary analyses have shown that these viruses were not reliably infecting cells at an MOI lower than 0.1. EILV MP458-NA-2018-PP reduced the replication rates of all superinfecting viruses albeit to a variable extent. An approximately 7-fold reduction in CHIKV genome copy numbers was observed at 1 dpi and a 2-fold reduction was measured the following days in cells pre-infected with MP458-NA-2018-PP compared to mock infected cells (Figure 3A). Presence of MP458-NA-2018-PP reduced SINV genome copy numbers about 10-fold at 1 dpi, around 100-fold at 2 dpi, and about 500-fold at 3 dpi (Figure 3B). These data were confirmed in a repeated independent experiment. WNV replication rates showed a 2 to 5-fold decrease starting at 2 dpi (Figure 3C) and BAGV genome copy numbers were reduced about 10-fold at 1 dpi and about 5-fold at 2 to 4 dpi (Figure 3D).

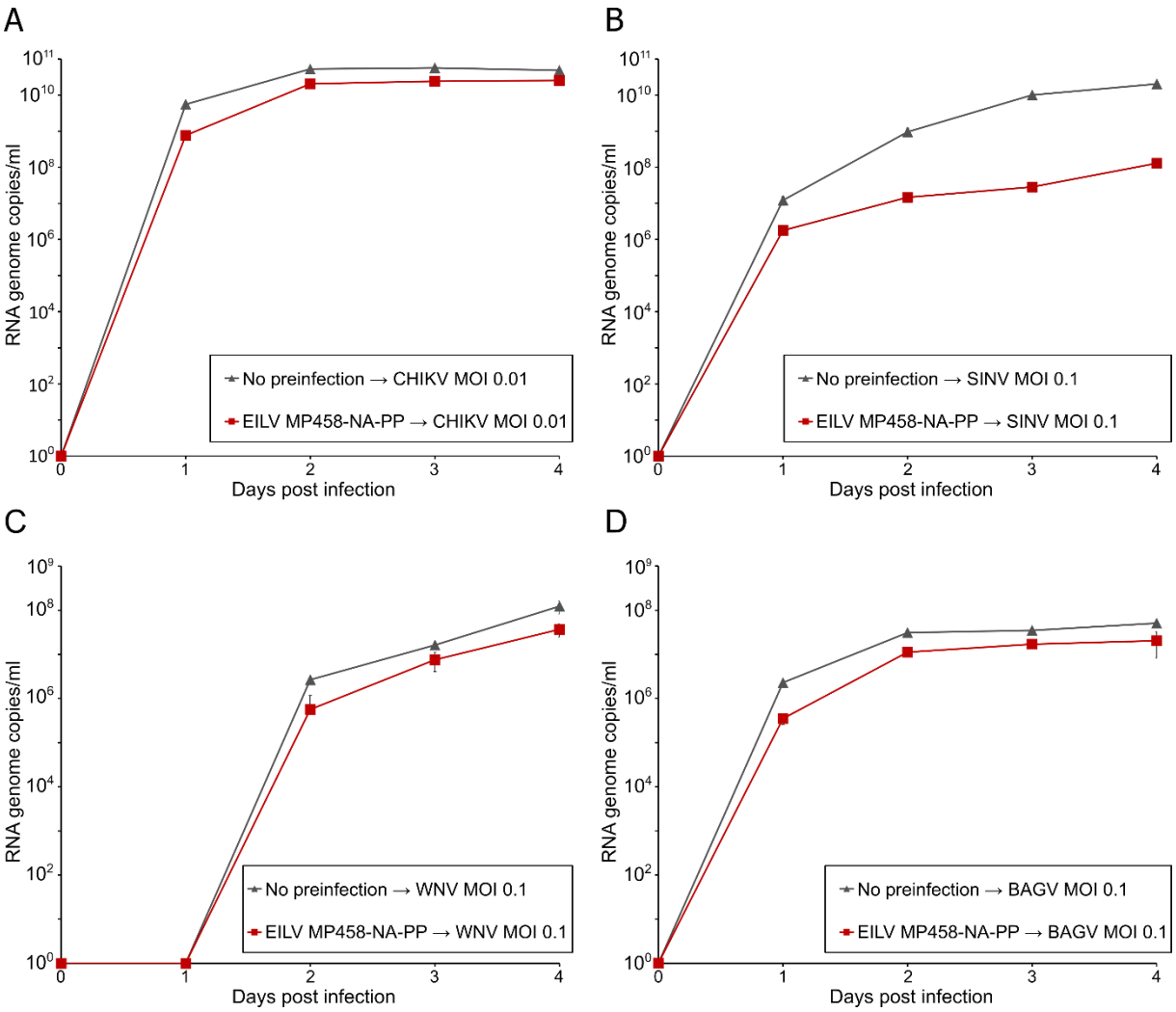

Figure 3: Superinfection experiments with EILV MP458-NA-2018 and different arboviruses. The mosquito cell line C7/10 (derived from *Aedes albopictus*) was infected with EILV MP458-NA-2018-PP in duplicates at an MOI of 1. After 16 hours, cells were super-infected with CHIKV (**A**), SINV (**B**), WNV (**C**), or BAGV (**D**) at the indicated MOIs. A sample of cell culture supernatant was taken every 24 hours for four consecutive days and viral copy numbers were determined by RT-qPCR.

Discussion

Alphaviruses with a host range restricted to insects are rarely detected in wild mosquito populations and represent a comparatively small group of viruses when compared to insect-specific viruses of other families. In this study, we detected and characterized an EILV variant isolated from mosquitoes from the Zambezi Region of north-eastern Namibia.

So far, EILV was found in *Anopheles coustani* mosquitoes from the Negev desert in Israel, a stone desert type landscape and in *Culex pipiens* mosquitoes collected in and around the Moroccan capital city of Rabat [7, 50]. In this study, EILV was isolated from *Culex univittatus* mosquitoes originating from Mudumu National Park, a shrub savanna type landscape, which is undisturbed from human cultivation for more than 40 years and is a state protected area [51]. Mosquitoes of the genera *Anopheles*, *Culex*, and *Aedes* have been shown to be susceptible for infection with EILV in laboratory experiments [8]. All EILV detections originate from landscapes with relatively hot and dry climate and a unimodal rainfall pattern [52-54]. We found a low infection rate of 0.098% (1/10,206 mosquitoes positive) which is in agreement with the low detection rates of insect-specific alphaviruses observed in other studies with the exception of EILV detection in Morocco [7, 12-15, 50].

EILV MP458-NA-2018 contained all the alphavirus-typical genetic elements also found in the original EILV strain EO329 from Israel. However, in the 3'-UTR we found a large 88 nt insertion that was not present in EO329. As the 3'-UTR of the Moroccan strain is not complete, we do not know how common such insertions are on EILV. We found poly(A) tails ranging in lengths from 11 to 17 Adenosine residues. From studies with polyadenylated viruses we know that the length of poly(A) tails is not always static, but can vary between quasispecies of one virus or between different points in the viral infection cycle [55-58]. Our data confirm the host range restriction of EILV to insects as concluded from previous studies [7, 9]. EILV MP458-NA-2018-PP did not replicate in the tested vertebrate cell lines and its replication was impaired at 31°C and completely blocked at 34°C [7].

ISVs have been shown to interfere *in vitro* and *in vivo* with arboviruses [16-21]. Here, we show that MP458-NA-2018-PP impairs the replication of vertebrate-pathogenic alpha- and flaviviruses. We studied the effects of MP458-NA-2018-PP on CHIKV and SINV that are both endemic in southern Africa, as well as on WNV and BAGV that were isolated from the same mosquito vector in the same region [37]. CHIKV and SINV superinfection exclusion had previously been reported in C7/10 cells pre-infected with EILV EO329 [18]. For SINV, a maximal 5,000-fold reduction of infectious particles was observed 1 dpi and no difference to non-pre-infected cells was observed 3 dpi in cells superinfected with EO329 [18]. In our study, we observed only a maximum 500-fold reduction in viral genome copies, but this was consistent up to 4 dpi. EILV EO329 also reduced rates in CHIKV particle production to a higher extend than MP458-NA-2018-

PP which was found to reduce CHIKV replication, but the effect of the latter isolate appeared to be more stable. The observed differences could be due to several reasons. MP458-NA-2018 and EO329 might differ in their phenotypic behavior, as well as different strains of CHIKV and SINV were used that may show difference in their susceptibility to interact with EILV [20, 59] and last but not least virus replication rates may differ from the production of infectious particles. Furthermore, we have demonstrated that MP458-NA-2018 leads to replicational reduction of two members of the genus *Flavivirus*, WNV and BAGV, which were previously found to be co-circulating in the Namibian Zambezi Region [37]. These data expand our knowledge on SIE of insect-specific alphaviruses to flaviviruses. As WNV and BAGV in the Zambezi Region were both detected in *Culex univittatus* mosquitoes, just like EILV MP458-NA-2018, an interaction of the different virus species in nature is imaginable. Nevertheless, the low EILV infection rate that was found in our study could make for rare encounters. Further experiments on the superinfection exclusion of WNV and BAGV, as well as of SINV and CHIKV, are needed to assess a possible effect *in vivo*. Although no other alphaviruses apart from EILV were found in this study, seroprevalence studies suggest the presence of SINV and CHIKV in the Zambezi Region [34-36].

Several ideas are being discussed how to exploit the unique features of ISVs for arbovirus control measures. As they can modulate the transmission of pathogenic arboviruses by mosquitoes, ISVs could be used in natural vector control strategies [60, 61]. Further, ISVs may also be used to reduce the fertility or fitness of insects by infecting them with specifically engineered ISVs [62, 63]. Moreover, ISVs can be used for the development of novel vaccines and safe to handle diagnostic antigens [10, 11, 64-70].

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Conflict of interest

The authors have declared that no competing interests exist.

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Chapter IV: Mosquito community composition in conservational areas in the Namibian Zambezi Region and its effect on associated RNA virus infections

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Abstract

The emergence of infectious disease has been connected to a number of environmental, ecological, demographical, and economic factors. Anthropogenic landscape transformation, like the generation of agricultural areas or urbanization, represents one of these factors that can lead to an increased amplification and dissemination of pathogens or put humans in increased contact with pathogens and their vectors. However, the exact mechanisms of this effect are not well understood yet.

In this work, the composition and structure of mosquito communities were studied in the Namibian Zambezi Region in Bwabwata and Mudumu National Parks and Mashi and Wuparo Conservancies across four different land-use types: farmland, grazing land, communally conserved pristine land, and governmentally conserved pristine land. Most sampling locations showed a high diversity of different mosquito species, which supported an equally high genetic diversity of RNA viruses from all the relevant families of mosquito viruses. Established arboviruses, like WNV, were identified among the virus detections. Further, we could detect a high number of putatively novel viruses, most of which were MSVs from the viral families *Flaviviridae* and *Phenuiviridae*. *Culex univittatus* and *Mansonia uniformis* mosquitoes were identified as the primary vectors of virus infections in the region. No conclusive correlation could be derived between mosquito host biodiversity and RNA virus infection prevalence. Instead, mosquito community composition was found to have a significant impact on the infection prevalence of the viruses studied.

Introduction

The emergence of an infectious disease is defined as the appearance of a pathogenic agent that has previously not been in broad contact with humanity [1]. A number of demographical, economical, ecological, and environmental factors have been identified as causative for the emergence of infectious diseases [2-6]. All these factors have the common mechanism of either promoting the amplification and/or dissemination of pathogens or putting humans in increased contact with pathogens and their vectors [7]. However, the re-emergence of infectious disease has historically been fueled by natural disasters, war, and a loss of social cohesion [8-11].

An important ecological impact on the emergence of infectious disease comes from anthropogenic landscape transformation, like the generation of agricultural areas or urbanization [4, 5, 12]. Here, the conversion of pristine land into human-used land (or vice versa, the renaturation of formerly used land) can have an influence on the transmission of infectious disease by changing breeding habitats, access to food resources, and transmission cycles [12, 13]. Famous examples include the amplification of *Plasmodium falciparum* and *Plasmodium vivax* (the pathogens causing malaria tropica and malaria tertiana, respectively) transmission by deforestation in the Amazon basin [14-17].

An important measure of the structure of an ecological system is its biodiversity [18]. In the dilution effect hypothesis, it is suggested that an increase in biodiversity in a biological system leads in turn to a decrease in the prevalence of pathogens, which has most notably been observed in studies on the tick-borne Lyme disease in northern America [19-21]. Species richness, which counts the absolute number of individual species, and Shannon diversity (represented by the letter “H”), which is defined as the negative sum of all the individual species proportions multiplied by the logarithmized individual species proportions ($H = -\sum_{i=1}^{n} pi \ln pi$), are among the most commonly used estimators for the diversity of a biological system [18, 22-25]. The community composition of an ecological system goes further into detail and describes the distribution of species abundances in an ecological system [26, 27].

The Zambezi Region is located in the northeasternmost extension of Namibia's national territory. Its vegetation is mostly made up of broadleaved woodland savanna and broadleaved tree-and-shrub savanna, interspersed by the grassland-type floodplains of the Zambezi, Kwando, Chobe, and Linyanti rivers [28]. The climate is defined as semi-tropical, with an alternating rainy season between October and April

and a dry season between May and September [28]. The terrestrial diversity is among the highest of all the parts of Namibia [28, 29]. The region is seeing rapid structural change and rising population growth rates, which are being promoted by the 2004 completion of the Walvis Bay–Ndola–Lubumbashi Development Corridor (WBNLDC) [30, 31]. The main drivers of land use change are agriculture, woodlogging, and cattle farming [29, 31-33]. Major activities in the region include large-scale green schemes like the Kalimbeza rice project and the Katima/Liselo farm project [34, 35]. Just recently, the local traditional authorities made over 23.890 ha of potentially cultivable land available for government development [35]. Two different approaches to nature conservation were taken in the region: state-run National Parks on the one hand, and Conservancies that practice community-based natural resource management (CBNRM) on the other [36]. National Parks make up around 28% of the region's total area, and Conservancies around 27% [37].

In this work, we studied the composition and structure of mosquito communities in the Namibian Zambezi Region across four different land-use types: farmland, grazing land, communally conserved pristine land, and governmentally conserved pristine land.

Methods

Sampling sites

All fieldwork was performed in the northeastern Namibian Zambezi Region. We selected Bwabwata and Mudumu National Parks and Mashi and Wuparo Conservancies for the collection of samples. Bwabwata National Park (further referred to as "Bwabwata NP") has been a conservation area since 1968 and has been known by its current name since 2007 [38, 39]. Its vegetation is dominated by Kalahari woodland, growing in deep aeolian Kalahari sand dunes [40]. After the relocation of local settlements in 1980, Mudumu National Park (further referred to as "Mudumu NP") was officially established in 1990 [41, 42]. Its vegetation is defined by mopane woodlands, harboring a mix of *Colophospermum mopane*, *Terminalia sericea*, and *Burkea africana* trees [43]. Sampling plots in the National Parks were selected for a balanced vegetational profile. In Mudumu NP, two sampling sites with varying elephant presences were selected. For this, one sampling site with a high indication of elephant presence (further referred to as "Mudumu NP high") and another sampling site with a

medium indication of elephant presence (further referred to as “Mudumu NP medium”) were selected. Mashi Conservancy (further referred to as “Mashi Cons.”) is mainly covered by Kalahari woodlands and, to a lesser extent, mopane woodlands [43, 44]. Wuparo Conservancy (further referred to as “Wuparo Cons.”) is a mix of mopane woodland (mostly) and grassland [43, 45]. The Namibian Conservancies usually divide their land into stock grazing and crop farming areas, areas labeled for residence, and conservation core areas, which are dedicated to tourism and hunting [46, 47]. The sampling plots in the agriculturally used areas of the Conservancies were selected to be in use for at least 10 years. In the core areas of Conservancies sampling plots were again selected for a balanced vegetational profile.

Mosquito sampling

Mosquitoes were sampled in Mudumu NP and Wuparo Cons. in November and December 2018, in Bwabwata NP in February and March 2019, and in Mashi Cons. in February and March 2020 [48]. CDC Gravid traps (John W. Hock Company, Gainesville, United States of America) and BG Sentinel traps (Biogents AG, Regensburg, Germany) were operated continuously, as were CDC Light traps (John W. Hock Company, Gainesville, United States of America) from dusk until dawn for five consecutive days at every sampling site (total trapping effort: 900 trap nights). BG Sentinel traps were baited with 1-Octen-3-ol, BG-Lure (Biogents AG, Regensburg, Germany), or worn socks on different days. CDC Light traps and BG Sentinel traps were additionally baited with CO_2 (250 grams of sugar mixed with 1 liter of water and 15 grams of yeast per 24 hours), released through a rubber hose attached to the opening of the traps. Trap points were set up on square-shaped plots with a 25-meter side length. Two CDC Light traps and two BG Sentinel traps were set up at the edges of the square facing each other, and one CDC Gravid trap was set up in the center of the square. One of the CDC Light traps was attached to a small tree or shrub at a height of around 1.7 meters, and the other CDC light trap was elevated to average canopy height with the help of a rope winch. We sampled mosquitoes on a total of twelve different plots, in Mudumu and Bwabwata NP (Kwando Core Area). In Wuparo and Mashi Cons., we selected and sampled eight plots each of Conservancy core area, grazing land, and farmland. The BG Sentinel traps and CDC Gravid traps were preferably set up in wind- and sun-shaded tree or shrub groves. Farmland sampling plots were placed at the edges of fields in order to also include one tree for the setup

of the canopy-height CDC Light trap. The combination of trap types and attractants was used to sample the highest possible diversity of mosquito species. Mosquitoes were stored in liquid nitrogen in the field until transportation to the laboratory facilities.

Mosquito identification

Mosquito species were individually identified in the laboratory by morphological features, using a stereomicroscope and the standard keys for the Afrotropical region [49-51].

Viral RNA extraction and PCR screening

Using a Tissue Lyser (QIAGEN, Hilden, Germany) and ceramic beads, individual mosquito specimens were homogenized in 500 µl of phosphate-buffered saline (PBS). 100 µl of the individual mosquito homogenates was then sorted into pools of 10 according to species and sampling site. The remaining mosquito homogenate was kept in order to do investigations on individual specimens. With the MagNA Pure 96 DNA and Viral NA Small Volume Kit, RNA was extracted from 200 µl of pooled mosquito homogenates (Roche Diagnostics, Mannheim, Germany) and eluted with 100 µl of supplied elution buffer. SuperScript IV reverse transcriptase from Thermo Fisher Scientific GmbH, Dreieich, Germany, and random hexamer primers (Integrated DNA Technologies Germany GmbH, Munich, Germany) were then used for the creation of cDNA. Mosquito pools were screened for mesoniviruses [52], orthobunyaviruses [53], phleboviruses [54], and rhabdoviruses [53] using generic PCR assays in accordance with published protocols. Nucleotide sequences, inferred by Sanger sequencing (Microsynth Seqlab GmbH, Göttingen, Germany), were analyzed with Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) and the GenBank Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/).

Phylogenetic analysis

Viral open reading frames (ORFs) were identified in Geneious and aligned with related sequences with the MAFFT algorithm [55]. All phylogenies were inferred using PhyML, using the Smart Model Selection (SMS) method [56, 57]. All phylogenetic trees were rooted on midpoint, and confidence testing was performed based on 1,000 bootstrap iterations.

Statistics

The mean values for the total abundance of individuals sampled on every plot were calculated and compared with each other for the different sampling sites (core area, farmland, and grazing land in Mashi and Wuparo Cons. and sampling sites in Bwabwata and Mudumu National Parks) using a one-way analysis of variance (ANOVA). The data was not transformed prior to analysis. Species accumulation curves were prepared for each land-use type by plotting the number of species collected against the sampled trap nights. A chi-square test was used to compare the distribution of different mosquito tribes and the sex of the sampled mosquitoes in the different land-use types. Rank abundance diagrams were created to study species dominance proportions. For this analysis, individuals that could not be determined on a species level were partitioned to the species of the respective subgenus, genus, or family according to their weighted abundance. ANOVA was used to determine a difference in mean species richness for the different land-use types. Student's *t*-tests were applied for pairwise comparison of the species richness of different sampling sites. The data was log-transformed prior to analysis, and a Bonferroni correction for multiple comparisons was used. ANOVA was further applied to compare differences in the mean Shannon diversity indices. Again, Student's *t*-tests were also used for pairwise comparison of the Shannon indices of different sampling sites. Data was not transformed prior to analysis, and again, a Bonferroni correction for multiple comparisons was used.

A nonmetric multidimensional scaling (NMDS) analysis was performed to assess differences in mosquito community composition and mosquito vector community composition. Both datasets were log (n+1) transformed prior to analysis. Monte Carlo permutation tests (9999 runs) and a Bonferroni correction were applied to examine the ordination axes and the different species for significant correlation. Permutational Multivariate Analysis of Variance (PERMANOVA) was used to check differences in mosquito community composition and mosquito vector community composition for significance [58, 59]. NMDS ordination analysis with the same preferences was also used to study differences in the virus community composition of the different sampling sites. The distribution of different virus families in the sampling sites was compared using the chi-square test.

Microsoft Excel version 2206 (Build 15330.20264 click to run edition) was used for the majority of statistical analyses, only NMDS and PERMANOVA was performed in Past 4.03 [60].

Results

Mosquito collection

A total of 9,301 mosquitoes were sampled across four different land-use types in national parks and conservancies in the Zambezi region in north-eastern Namibia. In field studies conducted between October 2018 and March 2020, a total sampling effort of 900 trap nights was achieved on 36 plots, with an overall number of 258 +/- 173 (mean +/- standard deviation) mosquitoes collected per plot. According to one-way ANOVA analysis, catch sizes were significantly differently distributed among the different land-use types (F = 2.99; degrees of freedom = 8; $P \leq 0.05$) (Figure 1). The sample data set was not standardized before further processing (equal-effort sampling).

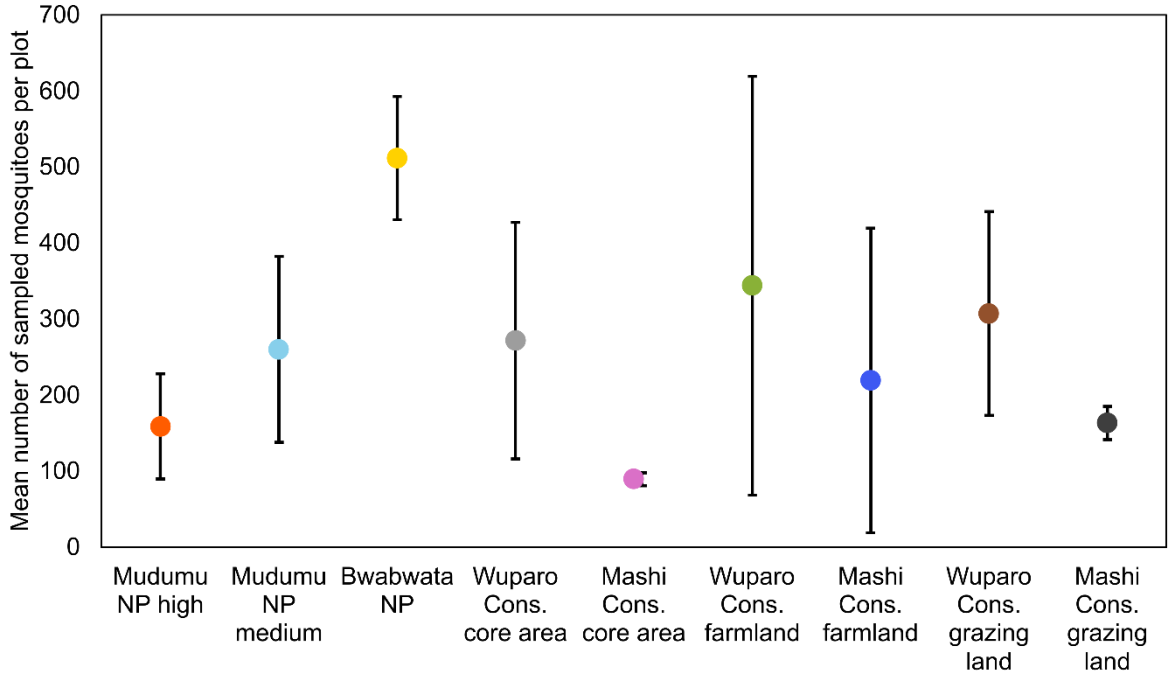

Figure 1: Mean number of sampled mosquitoes per plot. The arithmetic mean values for mosquitoes collected per sampling plot (n = 4) in the Namibian Zambezi Region in Bwabwata and Mudumu NP, and Mashi and Wuparo Cons. across four different land-use types, farmland, grazing land, communally conserved pristine land,

and governmentally conserved pristine land. According to one-way ANOVA there is a significant difference between the mean values (F = 2.99; degrees of freedom = 8; $P \leq 0.05$). Error indicators show standard deviation.

For evaluating the sampling range of the mosquito trapping, a species accumulation curve was created for each land-use type (Figure 2). The majority of the curves show an approximate pattern of strong initial growth with many species adding in the first quarter and asymptomatic flattening with few species appearing in the diagram's back half.

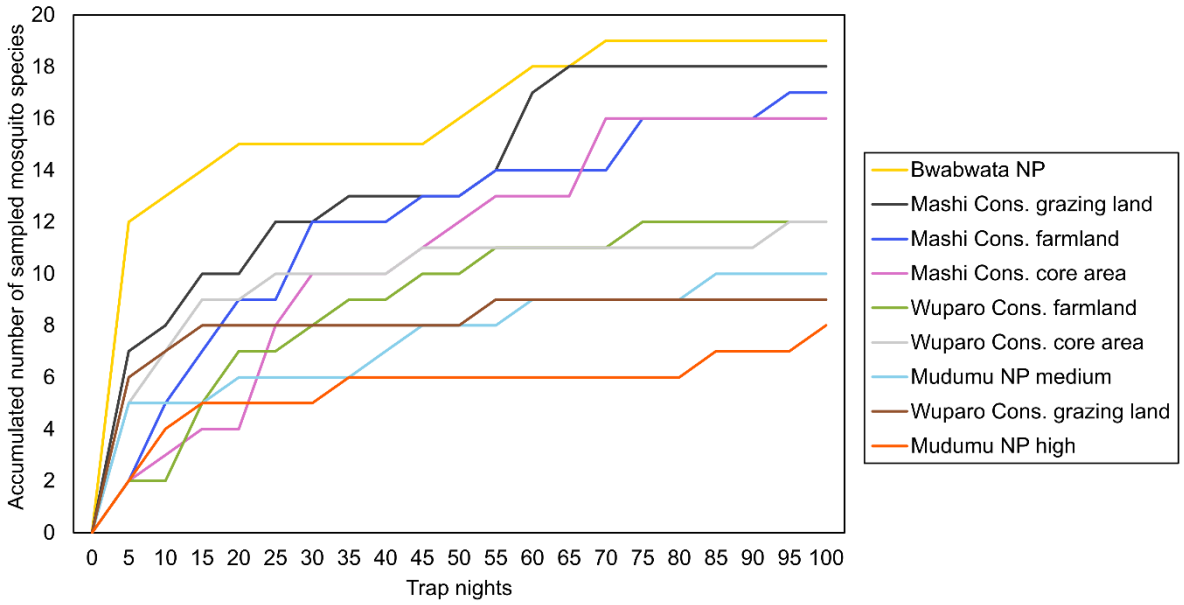

Figure 2: Species accumulation curve for sampled mosquitoes. For the evaluation of the total mosquito sampling effort, the number of collected species was plotted against the sampled trap nights for all sampling sites.

Analysis of mosquito community

Mosquitoes from the two subfamilies Anophelinae and Culicinae and six different tribes were found in the sample set. The distribution of subfamilies and tribes in the different sampling sites was evaluated using a chi-square test of equality. Members of the subfamily Anophelinae were predominantly found in grazing land and core area of Mashi Cons. and Bwabwata NP (χ^2 = 442.20; degrees of freedom = 8; $P \leq 0.001$), whereas Culicinae mosquitoes were overrepresented in all land-use types of Wuparo Cons. and farmland of Mashi Cons. (χ^2 = 20.81; degrees of freedom = 8; $P \leq 0.01$).

The majority of sampled mosquitoes belonged to the subfamily Culicinae (95.5%), while Anophelinae only made up 4.5% of the total catch (Table 1).

Table 1: Abundance of collected mosquitoes sorted by subfamily and sampling site.

	Mudumu NP high		Mudumu NP medium		Bwabwata NP		Wuparo Cons. core area		Mashi Cons. core area		Wuparo Cons. farmland		Mashi Cons. farmland		Wuparo Cons. grazing land		Mashi Cons. grazing land		Total		χ^2-Test	
	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	χ^2	P-Value
Anophelinae	25	3.94	75	7.21	152	7.43	13	1.20	34	9.50	4	0.29	6	0.68	2	0.16	107	16.39	418	4.49	442.20	< 0.001
Culicinae	610	96.06	965	92.79	1894	92.57	1074	98.80	324	90.50	1372	99.71	871	99.32	1227	99.84	546	83.61	8883	95.51	20.81	< 0.01

Mosquitoes of the Culicini tribe were significantly more abundant in Mudumu NP and Mashi Cons. (χ^2 = 1401.15; degrees of freedom = 8; $P \leq 0.001$), while mosquitoes of the Mansoniini tribe were strongly overrepresented in Bwabwata NP and Wuparo Cons. (χ^2 = 1690.45; degrees of freedom = 8; $P \leq 0.001$). Mosquitoes of the Aedini tribe were predominantly found in Mashi Cons. (χ^2 = 148.73; degrees of freedom = 8; $P \leq 0.001$) and mosquitoes of the Aedeomyiini tribe in Mudumu NP (χ^2 = 106.26; degrees of freedom = 8; $P \leq 0.001$). Mosquitoes of the tribes Culicini and Mansoniini comprised more than 90% of the sampled Culicinae subfamily (Table 2).

Table 2: Abundance of sampled Culicinae mosquitoes sorted by tribe and sampling site.

	Mudumu NP high		Mudumu NP medium		Bwabwata NP		Wuparo Cons. core area		Mashi Cons. core area		Wuparo Cons. farmland		Mashi Cons. farmland		Wuparo Cons. grazing land		Mashi Cons. grazing land		Total		χ^2-Test	
	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	χ^2	P-Value
Aedeomyiini	22	3.61	30	3.11	20	1.06	2	0.19	0	0.00	15	1.09	1	0.11	4	0.33	0	0.00	74	1.63	106.26	< 0.001
Aedini	0	0.00	9	0.93	94	4.96	52	4.84	36	11.11	30	2.19	64	7.35	75	6.11	50	9.16	155	3.41	148.73	< 0.001
Culicini	562	92.13	699	72.44	297	15.68	347	32.31	267	82.41	455	33.16	729	83.70	426	34.72	452	82.78	1905	41.93	1401.15	< 0.001
Ficalbiini	0	0.00	0	0.00	11	0.58	0	0.00	0	0.00	0	0.00	14	1.61	0	0.00	0	0.00	11	0.24	77.66	< 0.001
Mansoniini	20	3.28	202	20.93	1469	77.56	656	61.08	6	1.85	838	61.08	35	4.02	704	57.38	13	2.38	2347	51.66	1690.45	< 0.001
Uranotaeniini	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	0.18	0	0.00	15.27	NS
Tribe unknown	6	0.98	25	2.59	3	0.16	17	1.58	15	4.63	34	2.48	28	3.21	18	1.47	30	5.49	51	1.12	93.02	< 0.001

The ensemble of mosquitoes, collected from all plots taken together, harbored a total of 33 species belonging to 9 different genera. The catch numbers for the different sampling sites are summarized in Table 3.

Table 3: Abundance of sampled mosquitoes by species and sampling site.

	Mudumu NP high	Mudumu NP medium	Bwabwata NP	Wuparo Cons. core area	Mashi Cons. core area	Wuparo Cons. farmland	Mashi Cons. farmland	Wuparo Cons. grazing land	Mashi Cons. grazing land	Total N	Total %
Aedeomyia Aedeomyia africana	22	30	20	2	0	15	1	4	0	94	1.01
Aedes Aedimorphus hirsutus	0	0	15	0	6	28	4	57	19	129	1.39
Aedes Aedimorphus ochraceus	0	0	0	0	0	0	0	0	1	1	0.01
Aedes Aedimorphus sp.	0	0	0	0	0	0	1	0	0	1	0.01
Aedes Mucidus scatophagoides	0	0	0	0	0	0	1	0	0	1	0.01
Aedes Neomelaniconion circumluteolus	0	0	46	0	20	0	46	0	21	133	1.43
Aedes Pseudarmigeres argenteoventralis	0	0	0	0	1	0	0	0	0	1	0.01
Aedes Pseudarmigeres sp.	0	0	0	0	0	0	0	0	2	2	0.02
Aedes Stegomyia aegypti	0	0	1	0	2	0	1	0	3	7	0.08
Aedes Stegomyia chaussieri	0	0	1	0	0	0	0	0	0	1	0.01
Aedes Stegomyia luteocephalus	0	0	0	0	0	0	1	0	0	1	0.01
Aedes Stegomyia simpsoni	0	0	0	0	1	0	0	0	0	1	0.01
Aedes Stegomyia sp.	0	9	19	52	3	2	2	14	1	102	1.10
Aedes Zavortinkius sp.	0	0	1	0	0	0	0	0	0	1	0.01
Aedes sp.	0	0	11	0	3	0	8	4	3	29	0.31
Anopheles Cellia argenteolobatus	0	0	0	0	0	0	0	0	2	2	0.02
Anopheles Cellia rufipes	0	0	11	0	0	0	0	0	0	11	0.12
Anopheles Cellia schwetzi	0	0	0	0	0	0	1	0	0	1	0.01
Anopheles Cellia squamosus/cydippis	0	0	0	0	0	0	2	0	64	66	0.71
Anopheles Cellia sp.	0	0	0	0	1	0	0	0	0	1	0.01
Anopheles sp.	25	75	141	13	33	4	3	2	41	337	3.62
Coquillettidia Coquillettidia chrysosoma	0	0	4	0	0	0	0	0	0	4	0.04
Coquillettidia Coquillettidia fuscopennata	1	6	307	2	0	20	0	9	1	346	3.72
Coquillettidia Coquillettidia metallica	14	188	20	296	2	643	1	676	0	1840	19.78
Coquillettidia Coquillettidia sp.	0	0	1	5	0	1	0	0	0	7	0.08
Culex Culex antennatus	1	0	49	3	9	0	0	0	7	69	0.74
Culex Culex argenteopunctatus ssp. kingi	0	1	0	1	3	2	0	3	4	14	0.15
Culex Culex pipiens	0	0	0	0	34	0	32	0	29	95	1.02
Culex Culex quinquefasciatus	0	1	0	1	0	4	0	0	0	6	0.06
Culex Culex simpsoni	9	1	27	0	11	0	10	0	16	74	0.80
Culex Culex sp.	18	45	113	146	112	88	188	33	151	894	9.61
Culex Culex univittatus	534	646	41	194	94	357	451	390	224	2931	31.51
Culex Culiciomyia nebulosus Var. pseudocinereus	0	0	2	0	0	0	1	0	10	13	0.14
Culex Culiciomyia sp.	0	0	1	0	0	0	0	0	0	1	0.01
Culex culiciomyia semibrunneus	0	0	0	0	0	0	1	0	0	1	0.01
Culex Eumelanomyia rima	0	0	14	0	1	0	0	0	0	15	0.16
Culex Eumelanomyia sp.	0	0	1	0	0	0	0	0	0	1	0.01
Culex Oculeomyia poicilipes	0	5	1	1	1	1	3	0	3	15	0.16
Culex sp.	0	0	48	0	0	0	43	0	8	99	1.06
Culicinae	6	25	3	17	15	34	28	18	30	176	1.89
Ficalbia Etorleptiomyia mediolineata	0	0	6	0	0	0	10	0	0	16	0.17
Ficalbia sp.	0	0	5	0	0	0	4	0	0	9	0.10
Lutzia Metalutzia tigripes	0	0	0	1	2	3	0	0	0	6	0.06
Mansonia Mansonioides africana nigerrima	0	0	179	0	0	0	0	0	1	180	1.94
Mansonia Mansonioides uniformis	5	8	958	353	4	174	32	19	11	1564	16.82
Mansonia Mansonioides sp.	0	0	0	0	0	0	2	0	0	2	0.02
Uranotaenia sp.	0	0	0	0	0	0	0	0	1	1	0.01

The genera *Aedes* and *Culex* both showed the highest diversities, with a total of ten different species collected on all plots taken together. Most abundantly sampled were the genera *Culex* (total number of 4228; 45.46%) and *Mansonia* (total number of 3943; 42.39%). Most numerous among the encountered species were *Culex univittatus* (2931; 31.51%), *Coquillettidia metallica* (1840; 19.78%), and *Mansonia uniformis* (1564; 16.82%). Only the two mosquito species, *Culex univittatus* and *Mansonia uniformis*, were encountered throughout all the sampling sites; the rest of the species were confined to a maximum of 8 different sampling sites. Bwabwata NP and Mashi Cons. farmland sampling sites had the highest number of unique species, followed by Mashi Cons. grazing land and Mashi Cons. core area. More than three quarters of the overall catch consisted of female mosquitoes (7218; 77.60%), and around one fifth

represented male mosquitoes (1900; 20.43%). For 183 (1.97%) individual mosquito specimens, sex could not be determined because of bad sample conditions (Table 4). Male mosquitoes were significantly more abundant in catches from Wuparo Cons. and Bwabwata NP ($\chi^2 = 559.66$; degrees of freedom = 8; $P \leq 0.001$).

Table 4: Abundance of mosquito sexes in the different sampling sites.

	Mudumu NP high		Mudumu NP medium		Bwabwata NP		Wuparo Cons. core area		Mashi Cons. core area		Wuparo Cons. farmland		Mashi Cons. farmland		Wuparo Cons. grazing land		Mashi Cons. grazing land		Total		χ^2-Test	
	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	χ^2	P-Value
Female mosquitoes	585	92.13	881	84.71	1663	81.28	566	52.07	299	83.52	915	66.50	800	91.22	918	74.69	591	90.51	7218	77.60	178.70	< 0.001
Male mosquitoes	40	6.30	132	12.69	370	18.08	464	42.69	58	16.20	419	30.45	65	7.41	297	24.17	55	8.42	1900	20.43	559.66	< 0.001
No sex determinable	10	1.57	27	2.60	13	0.64	57	5.24	1	0.28	42	3.05	12	1.37	14	1.14	7	1.07	183	1.97	102.31	< 0.001

A rank abundance analysis was performed to further investigate the species dominance in the different mosquito communities of the sampling sites (Figure 3). The catch from sampling sites in Mudumu NP and Mashi Cons. was strongly dominated by a single species, *Culex univittatus*. In Bwabwata NP, there was a single dominant species as well, *Mansonia uniformis*. The samples from Wuparo Cons. share two dominant species, *Coquillettidia metallica* and *Culex univittatus*, and in the core area sampling sites there is an additional third dominant species, *Mansonia uniformis*.

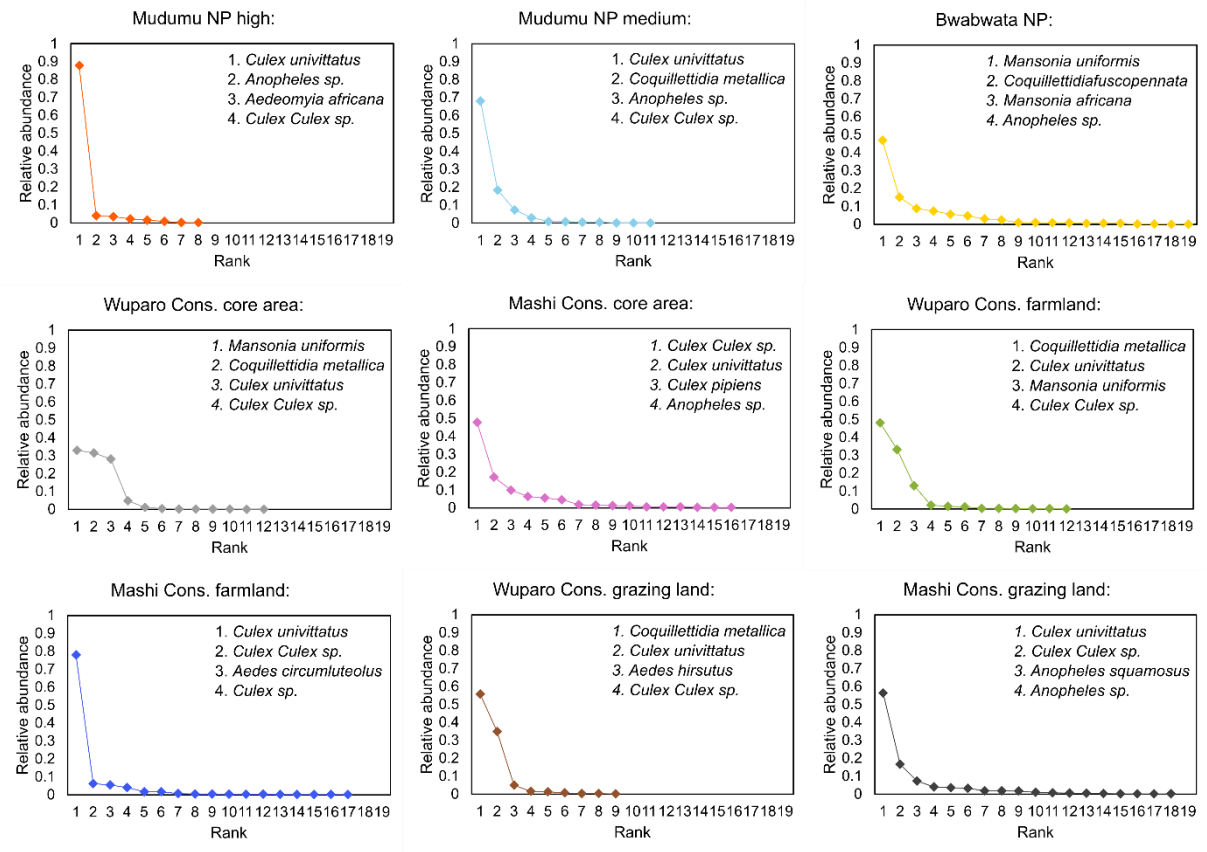

Figure 3: Rank abundance diagrams for all sampling sites. Species dominance in the mosquito communities of the different sampling sites was investigated by rank abundance analysis. The four most abundant mosquito species are given for each sampling site. *Culex univittatus* predominated in the sampling sites in Mudumu NP and Mashi Cons.. In Bwabwata NP, *Mansonia uniformis* is mainly shaping the ensemble. *Coquillettidia metallica* and *Culex univittatus* were highly represented in all Wuparo Cons. sampling sites, while *Mansonia uniformis* was only found in large numbers in Wuparo Cons. core area.

Biodiversity indices

Species richness was calculated for every plot, and means were compared among the different land-use types. Mosquito species richness differed significantly between the habitat types (one-way ANOVA: $F = 13.62$; degrees of freedom = 8; $P \leq 0.000001$) (Figure 4). Pairwise comparison showed significantly higher mean species richness in Bwabwata NP than in the Mudumu NP high and medium sampling sites (Student's *t*-test: *t*-statistic = 18.93; degrees of freedom = 6; $P \leq 0.000001$ and *t*-statistic = 5.39; degrees of freedom = 6; $P \leq 0.001$). The comparison of core area, farmland, and grazing land sampling sites in Mashi and Wuparo Cons. only showed a higher mean

species richness for grazing land sampling sites in Mashi Cons. (Student's *t*-test: *t*-statistic = 2.81; degrees of freedom = 6; $P > 0.01$ for core area; *t*-statistic = 1.71; degrees of freedom = 6; $P > 0.01$ for farmland; *t*-statistic = 5.77; degrees of freedom = 6; $P \leq 0.001$ for grazing land). The different sampling sites within the Conservancies showed no significant variation (one-way ANOVA: $F = 2.67$; degrees of freedom = 2; $P > 0.05$ for Mashi Cons.; $F = 0.20$; degrees of freedom = 2; $P > 0.05$ for Wuparo Cons.).

Shannon diversity indices were calculated for all plots as an additional indicator for mosquito community biodiversity. The comparison of mean Shannon indices by one-way ANOVA analysis suggested significant differences ($F = 8.12$; degrees of freedom = 8; $P \leq 0.0001$) (Figure 5). Again, the sampling site in Bwabwata NP showed a higher Shannon index than the sampling sites in Mudumu NP (Student's *t*-test: *t*-statistic = 8.45; degrees of freedom = 6; $P \leq 0.0001$ for Mudumu NP high; *t*-statistic = 9.92; degrees of freedom = 6; $P \leq 0.0001$ for Mudumu NP medium). The comparison of core area, farmland, and grazing land sampling sites in Mashi and Wuparo Cons. showed no significant differences in Shannon indices (Student's *t*-test: *t*-statistic = 1.80; degrees of freedom = 6; $P > 0.01$ for core area; *t*-statistic = 0.36; degrees of freedom = 6; $P > 0.01$ for farmland; *t*-statistic = 2.34; degrees of freedom = 6; $P > 0.01$ for grazing land). Only the sampling sites within Wuparo Cons. showed significant differences (one-way ANOVA: $F = 1.52$; degrees of freedom = 2; $P > 0.05$ for Mashi Cons.; $F = 9.60$; degrees of freedom = 2; $P \leq 0.01$ for Wuparo Cons.).

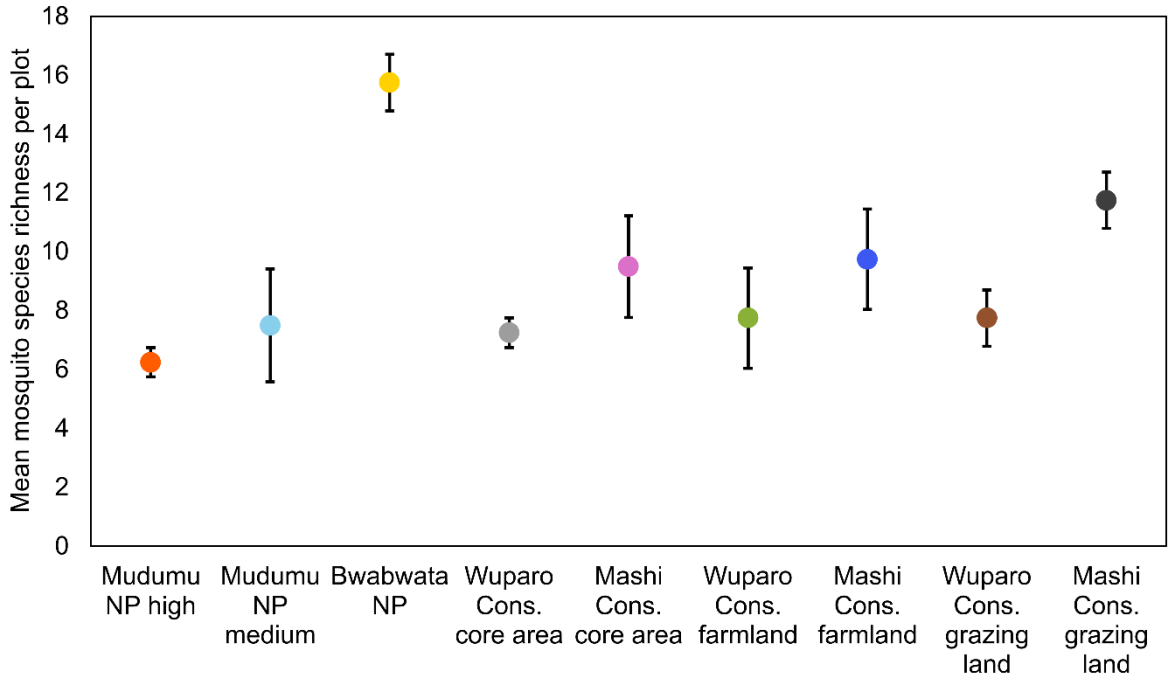

Figure 4: Mean number of mosquito species richness per plot. The arithmetic mean values for species richness per sampling plot (n = 4) in National Parks, Conservancy core areas, farmland, and grazing land in the Namibian Zambezi Region. There is a significant difference between the mean values, according to one-way ANOVA (one-way ANOVA: $F = 13.62$; degrees of freedom = 8; $P \leq 0.000001$). Error indicators show standard deviation.

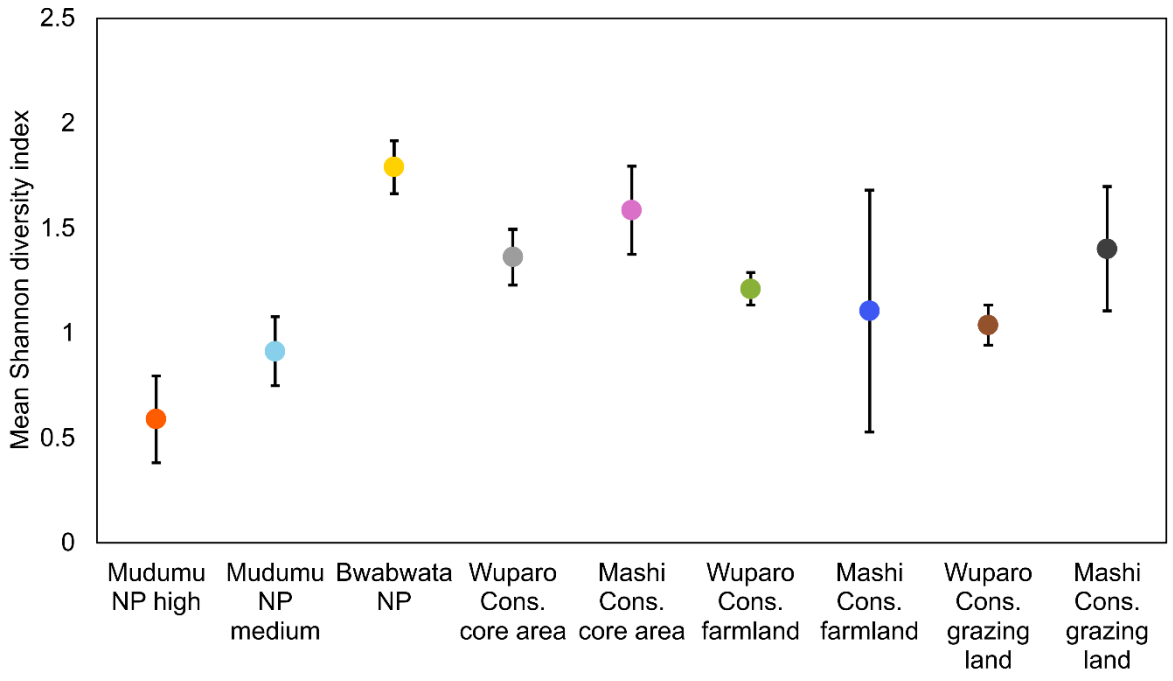

Figure 5: Mean Shannon diversity indices. The arithmetic mean values of the

Shannon diversity index per sampling plot in National Parks, Conservancy core areas, farmland, and grazing land in the Namibian Zambezi Region. The higher the Shannon index, the higher the diversity it represents. There is a significant difference between the mean values, according to one-way ANOVA (F = 8.12; degrees of freedom = 8; P ≤ 0.0001). Error indicators show standard deviation.

Ordination Analysis

Mosquito community composition was further studied using an NMDS ordination analysis. Axis 1 (capturing 55% of the variation) of the ordination analysis clearly divided the Bwabwata NP sampling sites from the rest of the sampling sites, and Axis 2 (capturing 24% of the variation) separated the sampling sites in Wuparo Cons. from the sites in Mashi Cons. (NMDS stress value = 0.1027) (one-way PERMANOVA: *F* = 11.36; degrees of freedom = 8; *P* ≤ 0.001) (Figure 6). A total of 10 out of the 33 species examined showed a significant correlation with those two ordination axes (Table 5). *Anopheles rufipes*, *Coquillettidia fuscopennata*, *Culex antennatus*, *Culex rima*, Mansonia uniformis, and *Mansonia africana nigerrima* were attributed to Bwabwata NP. *Culex univittatus* mosquitoes were associated with Mudumu NP, Mashi Cons., and Wuparo Cons.. *Coquillettidia metallica* was mainly connected to Wuparo Cons., and *Aedes circumluteolus* and *Culex pipiens* were mainly connected to Mashi Cons. sampling sites.

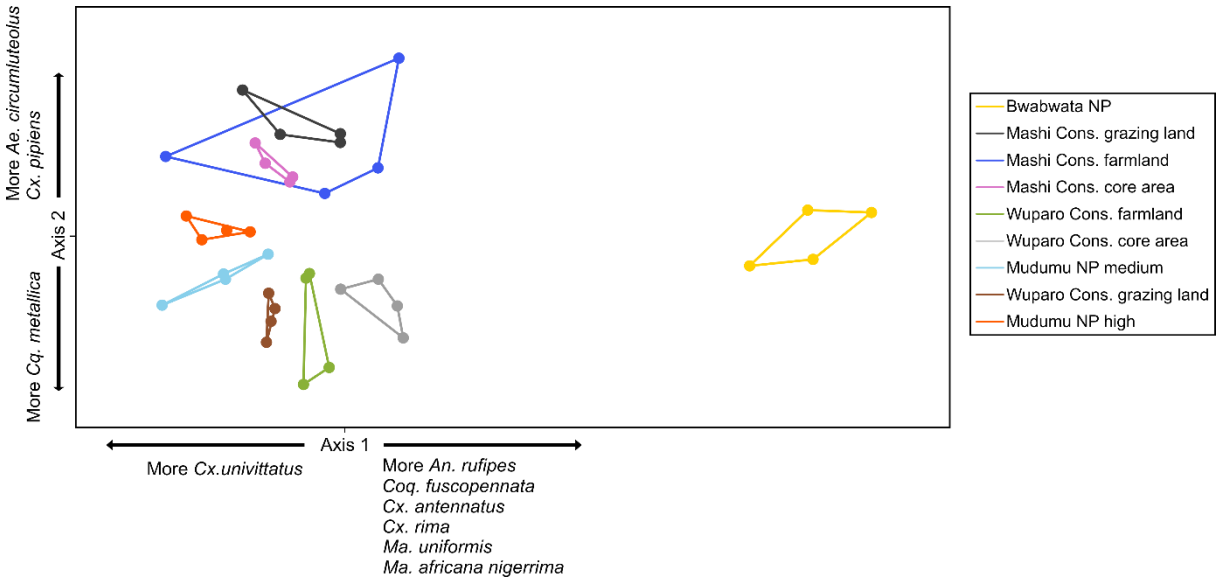

Figure 6: Ordination analysis of the mosquito community composition. In NMDS ordination analysis, Axis 1 showed clear differences between the sampling sites in

Bwabwata NP on the right side of the graph and the rest of the sites. Axis 2 separated the Wuparo Cons. sampling sites on the bottom side from the Mashi Cons. sampling sites on the top side of the diagram.

To examine differences in the composition of mosquito disease vectors, an NMDS analysis was performed on a subset of data, only taking into account the medically important mosquito species as provided by the Walter Reed Biosystematics Unit (WRBU) (http://www.wrbu.org/mqID/keysMQAlpha.html). Analysis of the vector community showed similar results to the NMDS analysis of the whole mosquito community (Figure 7). A total of nine medically important mosquito species significantly correlated with ordination Axis 1 and Axis 2, which respectively captured 53% and 25% of the total variation (NMDS stress value = 0.1046) (Table 5). A one-way PERMANOVA confirmed highly significant differences in the composition of vector communities in the different sampling sites (F = 13.23; degrees of freedom = 8; $P \leq 0.001$).

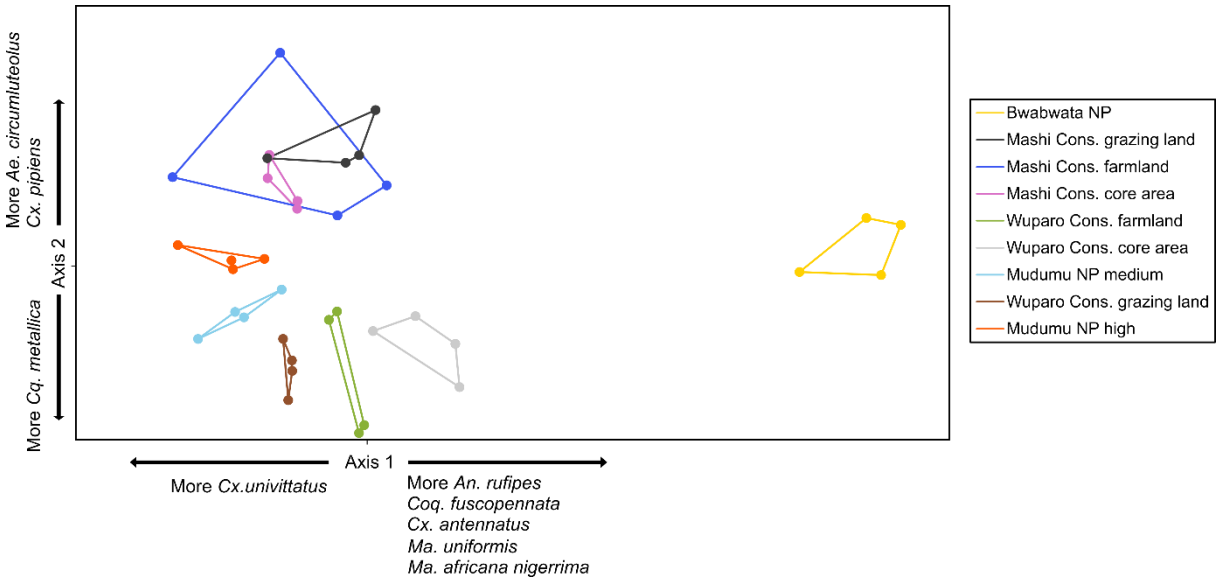

Figure 7: Ordination analysis of the mosquito disease vector community composition. The NMDS ordination analysis of the mosquito disease vector community showed similar results as the analysis done for the whole mosquito community. Axis 1 clearly divided the sampling sites in Bwabwata NP on the right side of the graph from the rest of the sites. Axis 2 showed differences between the Wuparo Cons. sampling sites on the bottom side and Mashi Cons. sampling sites on the top side of the diagram.

Table 5: Pearson correlation coefficients with two NMDS ordination axes of the significantly correlated mosquito species. The correlation coefficients, which were highly significant (P ≤ 0.001), using a Monte Carlo permutation test and a Bonferroni correction, are depicted in bold letters. For the total mosquito community analysis, all sampled species were used. Only the medically important mosquito species (as defined by WRBU) were considered for the mosquito vector community analysis.

	Analysis of total mosquito community				Analysis of mosquito vector community			
	Axis 1 coefficient	P-value	Axis 2 coefficient	P-value	Axis 1 coefficient	P-value	Axis 2 coefficient	P-value
Aedes circumluteolus	0.5360	0.0016	0.7469	**0.0001**	0.47166	0.0039	0.7821	**0.0001**
Anopheles Cellia rufipes	0.8744	**0.0001**	-0.0015	0.9939	0.87898	**0.0001**	0.071424	0.6773
Coquillettidia Coquillettidia fuscopennata	0.8094	**0.0001**	-0.3182	0.0546	0.82611	**0.0001**	-0.25066	0.1377
Coquillettidia Coquillettidia metallica	-0.0989	0.5767	-0.9292	**0.0001**	-0.043122	0.8121	-0.94071	**0.0001**
Culex Culex antennatus	0.6917	**0.0002**	0.2821	0.0913	0.67809	**0.0002**	0.32515	0.0513
Culex Culex pipiens	-0.1687	0.3407	0.8456	**0.0001**	-0.23733	0.167	0.82432	**0.0001**
Culex Culex univittatus	-0.6271	**0.0001**	-0.2201	0.2011	-0.64774	**0.0001**	-0.25975	0.1309
Culex Eumelanomyia rima	0.7113	**0.0001**	0.1055	0.5583				
Mansonia Mansonioides africana nigerrima	0.9263	**0.0001**	0.0082	0.9621	0.92551	**0.0001**	0.085799	0.6222
Mansonia Mansonioides uniformis	0.7906	**0.0001**	-0.3959	0.0172	0.81249	**0.0001**	-0.34059	0.0448

Genetic and phylogenetic analysis of the virus community

After morphological identification, mosquito species were homogenized individually and sorted into 1,021 pools, which were further combined into 130 super-pools. In total, RT-PCR screening revealed a total of 304 viral sequence fragments associated with seven different virus families: *Flaviviridae*, *Mesoniviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae*. Genetic and phylogenetic characterization of virus detections from RT-PCR screenings for *Flaviviridae* [48], *Reoviridae* [61], and *Togaviridae* (Guggemos et al., to be published) have previously been reported.

Peribunyaviridae

The family *Peribunyaviridae* currently holds four approved viral genera: *Orthobunyavirus*, *Herbevirus*, *Pacuvirus*, and *Shangavirus*. [62]. While the genera *Herbevirus* and *Shangavirus* harbor insect-specific viruses, *Pacuvirus* and *Orthobunyavirus* are mostly arthropod-transmitted viruses that can infect a range of vertebrates [62-67]. Especially the genus *Orthobunyavirus* harbors several viruses of high medical importance, that pose a potential threat to humans and their livestock [68-73]. They are mostly transmitted by mosquitoes of the genera *Culex* and *Ochlerotatus* and biting midges of the genus *Culicoides* [74, 75].

Screening of the mosquito samples for members of the *Peribunyaviridae* viral family yielded two novel virus species. Two strains of a novel peribunyavirus closely related

to Hangzhou sesamia inferens peribunyavirus 1 (HSIV-1), that was provisionally named Peribunya I (pairwise amino acid identities of 93.33% and 94.43% to HSIV-1 isolate DM1FY54970 (MZ209644)). Further, one strain of a novel orthobunyavirus related to Koongol virus (KOOV) and Wongal virus (WONV) was detected and provisionally named Peribunya II (pairwise amino acid identities of 87.32% to both, KOOV strain MRM31 (KP792669) and WONV strain MRM168 (MK896432)). Pairwise amino acid identities of less than 96% were applied for species demarcation, as suggested by the International Committee on Taxonomy of Viruses (ICTV) for the genus *Orthobunyavirus* [62].

In a phylogeny derived from various sequences of the *Peribunyaviridae* viral family, Peribunya I grouped with HSIV-1 on a long, solitary branch, in sister clade position to the members of the genus *Herbevirus* (Figure 8). Peribunya II, however, was placed within sequences of the genus *Orthobunyavirus*, forming a monophyletic clade with KOOV and WONV.

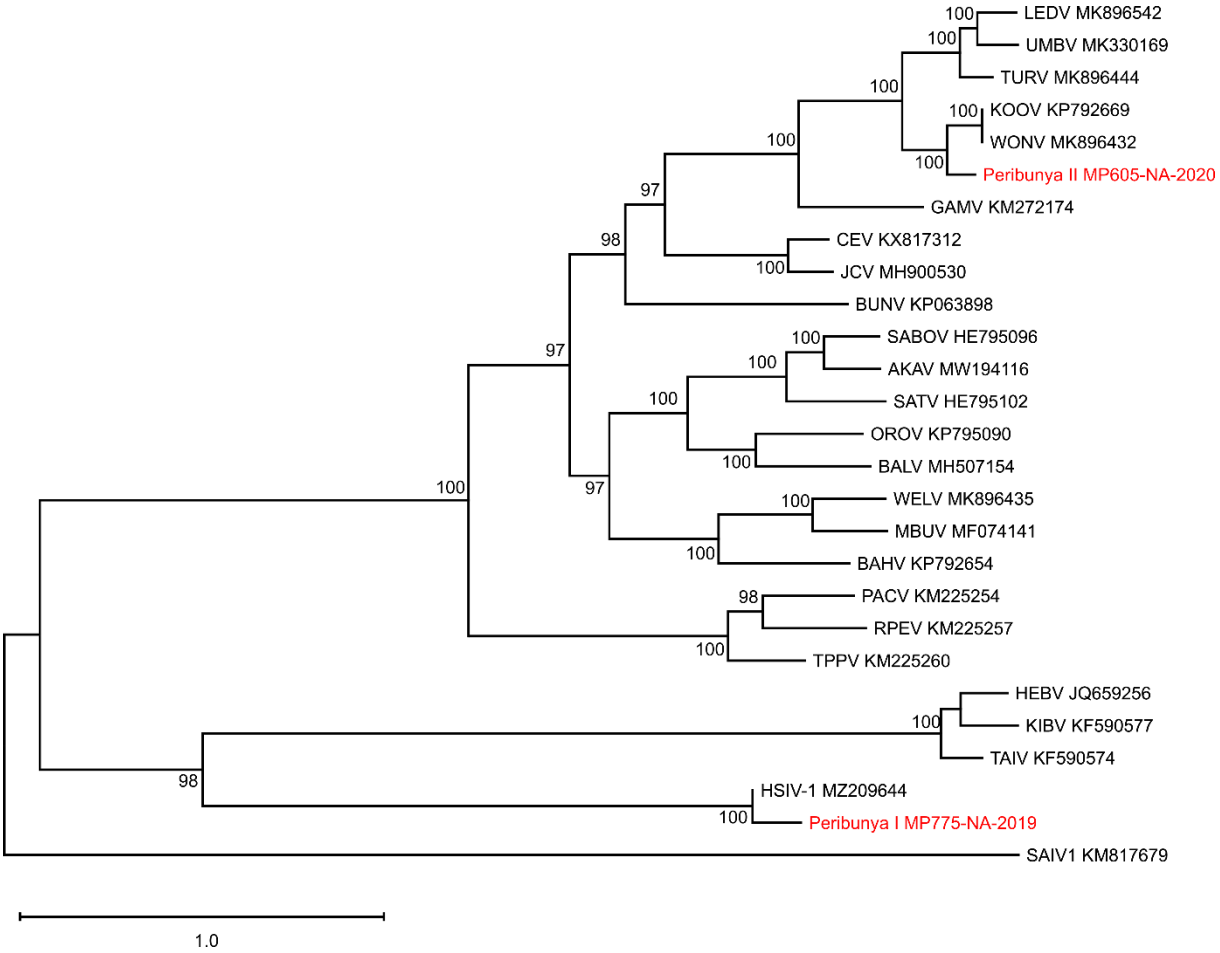

Figure 8: Phylogeny of detected members of the viral genus *Peribunyaviridae*. A maximum likelihood phylogenetic tree was derived for a protein alignment of viral L segments, coding for the RdRP. The phylogeny was calculated with the Q.yeast model,

using an estimated gamma shape parameter of 1.062 and an estimated proportion of 0.059 invariable sites, as selected by Smart Model Selection in PhyML (SMS). The phylogenetic tree was midpoint rooted, and percentual support values are given for each node, based on 1,000 bootstrap iterations. Only support values higher than 70% are shown. The virus detections from this publication, Peribunya I and II, are depicted in red coloring, and reference sequences accessed from GenBank are supplied with the respective accession number. The abbreviations for the reference viruses are as follows: AKAV = Akabane virus; BAHV = Bahig virus; BALV = Balagodu virus; BUNV = Bunyamwera virus; CEV = California encephalitis virus; GAMV = Gamboa virus; HSIV-1 = Hangzhou Sesamia Inferens peribunyavirus 1; HEBV = Herbert virus; JCV = Jamestown Canyon virus; KIBV = Kibale virus; KOOV = Koongol virus; LEDV = Lednice virus; MBUV = Mburo virus; OROV = Oropouche virus; PACV = Pacui virus; RPEV = Rio Preto da Eva virus; SABOV = Sabo virus; SAIV1 = Shuangao Insect virus 1; SATV = Sathuperi virus; TAIV = Taï virus; TPPV = Tapirape virus; TURV = Turlock virus; UMBV = Umbre virus; WELV = Weldona virus; WONV = Wongal virus.

Phenuiviridae

The family *Phenuiviridae* currently harbors 20 genera of viruses, infecting members of the kingdoms Animalia, Plantae, and Fungi [76-80]. Only members of the genera *Phlebovirus* and *Bandavirus* are known to infect vertebrates [76, 81-88]. They are primarily transmitted by mosquitoes, sandflies, and ticks [81, 83, 84, 88-92]. Besides, the family holds a number of insect-specific viruses, mainly in the genera *Phasivirus* and *Goukovirus* [93-96].

RT-PCR screening for members of the viral family *Phenuiviridae* revealed fragments of seven novel and two previously known virus species. Two strains of the insect-specific Gouléako virus (GOUV) (genus *Goukovirus*) (pairwise amino acid identities of 97.80%–97.92% to GOUV strain A5/CI/2004 (HQ541738)), which was provisionally named Phenui V, and six genetically divergent strains of the mosquito-isolated Fitzroy crossing tenui-like virus 1 (FCTenV1) (proposed genus *Tenuivirus*) (pairwise amino acid identities of 86.10%–91.82% to FCTenV1 isolate FCTeLV1/pool-6 (MT498812)), provisionally named Phenui I, were detected [94, 97]. For the genus *Tenuivirus*, a divergence of at least 15% in amino acid sequences has been proposed by ICTV for species demarcation [98].

Additionally, two novel viruses, related to the members of the genus *Goukovirus*, GOUV, Yichang Insect virus (YCV), and Cumuto virus (CUMV) were detected. Five strains of a novel virus, provisionally named Phenui II (pairwise amino acid identities of 68.42%–70.15% to GOUV strain A5/CI/2004 (HQ541738), 66.10%–70.69% to YCV strain YCYC01 (KM817703), and pairwise amino acid identities of 56.82%–67.24% to CUMV strain TR7904 (KF543244)). Two strains of a novel virus, provisionally named Phenui VI (pairwise amino acid identities of 58.06%–60.67% to GOUV strain A5/CI/2004 (HQ541738), 59.68%–62.19% to YCV strain YCYC01 (KM817703), 66.13%–66.74% to CUMV strain TR7904 (KF543244), and 58.62% to Phenui II strain MP158-NA-2018). For the genus *Goukovirus*, a minimum divergence of 10% in the polymerase core domain was proposed for the definition of a novel species [94, 99].

Further, four novel viruses, related to the members of the genus *Phasivirus* were found. Two strains of a novel virus related to Phasi Charoen-like phasivirus (PCLV), named Phenui III (pairwise amino acid identities of 84.28%–86.06% to PCLV isolate C302F/2016/BR (MN692603)). Two strains of another novel virus related to PCLV and Phenui III, which was provisionally named Phenui VII (pairwise amino acid identities of 82.61%–83.22% to PCLV isolate C302F/2016/BR (MN692603) and 89.61%–89.71% to Phenui III strain MP100-NA-2018). One strain of a novel virus related to Badu phasivirus (BADUV) and Parry's Creek phasivirus 1 (also named Kimberley phasivirus) (PCPhasV1) was provisionally named Phenui VIII (pairwise amino acid identities of 86.23% to BADUV isolate TS6347 (KT693187) and 84.06% to PCPhasV1 isolate PCPV1/pool-10 (MT498816)). And lastly, one strain of a novel virus, related to Cimo phenuivirus IV (CimoPV-IV) and Guadeloupe mosquito phasivirus (GMPV), provisionally named Phenui IX (pairwise amino acid identities of 73.33% to CimoPV-IV strain CimoPhV-IV/B98-CI-2004 (MZ202294) and 71.33% to GMPV strain Ab-AAF-1-3 (MN053784). For members of the genus *Phasivirus*, a minimum amino acid divergence of 10% is mostly being used for the definition of novel virus species [97, 100].

Further, one strain of a novel virus, related to the fly (Diptera) virus Hubei bunya-like virus 1 (HbBLV-1) (genus unassigned), was found and provisionally named Phenui IV (pairwise amino acid identities of 44.08% to HbBLV-1 strain SCM51446 (KX884804)) [101].

In the phylogenetic analysis of the detected phenuiviruses, Phenui II, Phenui VI, and Phenui V clustered within the *Goukovirus* phylogenetic clade (Figure 9). Phenui II

loosely grouped with YCV, Phenui VI with CUMV, and Phenui V closely aligned with GOUV. Phenui III, Phenui VII, Phenui VIII, and Phenui IX fell into the phylogenetic clade formed by the members of the genus *Phasivirus*. Phenui III and Phenui VII grouped together with PCLV and Phenui IX clustered basal to GMPV. Phenui VIII was placed in basal position to the group formed by BADUV and PCPhasV1. Phenui I closely grouped with FCTenV1 within the *Tenuivirus* phylogenetic clade. Phenui IV clustered basal to the unassigned group formed by Norway phlebovirus 1 (NWPV1) and Blacklegged tick phleboviruses 1 and 3 (BLTPV1 and BLTPV3), although with very low branch node support.

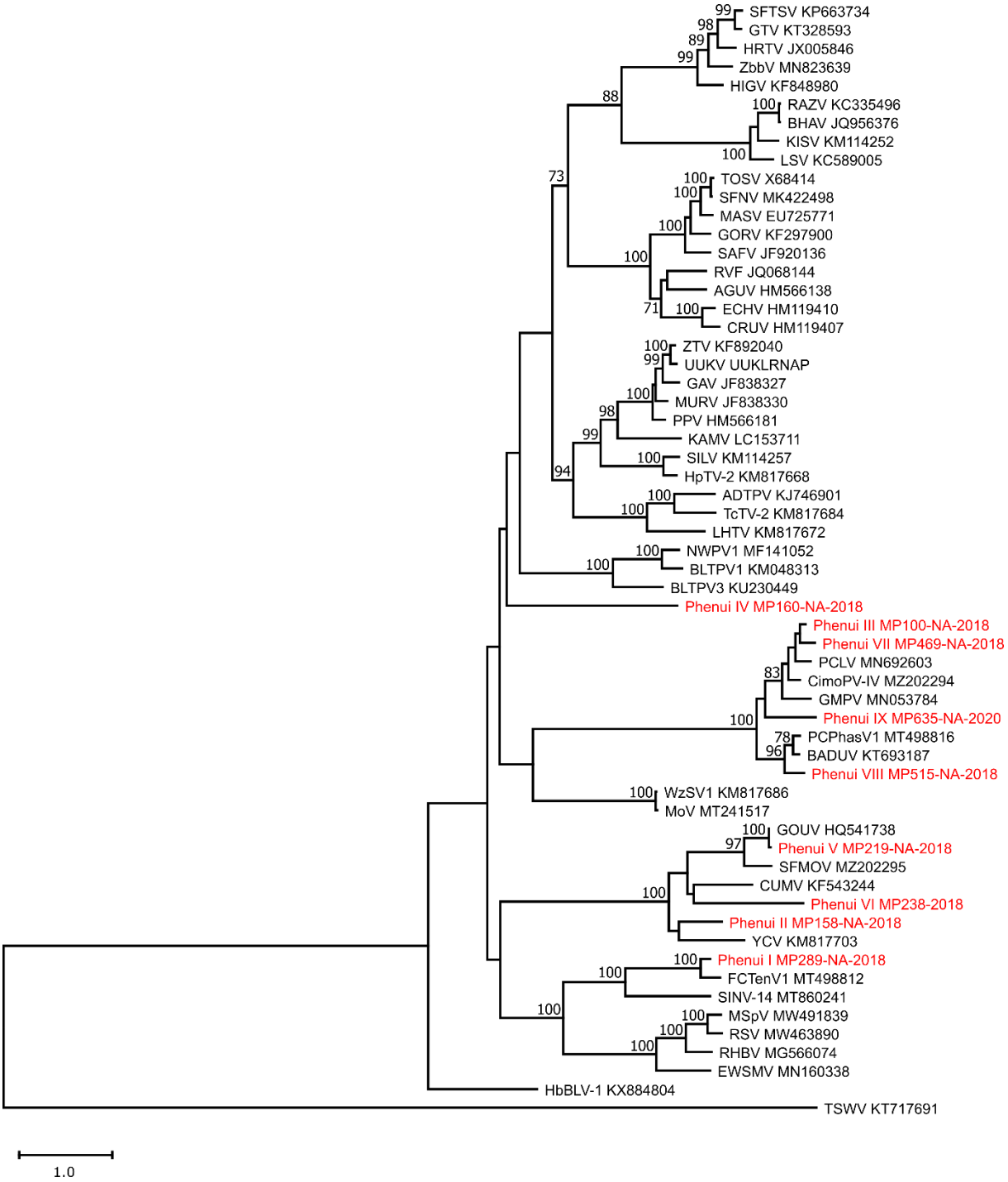

Figure 9: Phylogeny of detected phenuiviruses. A maximum likelihood phylogenetic tree was derived for a protein alignment of viral L segments, coding for the RdRP. The phylogenetic tree was inferred with the Q.yeast model and an estimated gamma shape parameter of 1.177 and an estimated proportion of 0.037 invariable sites, as chosen by the Smart Model Selection in PhyML (SMS). The phylogeny was midpoint rooted, and support values are shown for each branch node, on the basis of 1,000 bootstraps iterations. Only support values higher than 70% are shown. Virus detections from this publication, Phenui I-IX, are shown in red color, and reference sequences from

GenBank are complemented with their respective accession numbers. In addition to members of the family *Phenuiviridae*, tomato spotted wilt orthotospovirus of the viral family *Tospoviridae* was added to the alignment. Abbreviations for reference viruses are as follows: AGUV = Aguacate virus; ADTPV = American dog tick phlebovirus; BADUV = Badu phasivirus; BHAV = Bhanja virus; BLTPV1 = Blacklegged tick phleboviruses 1; BLTPV3 = Blacklegged tick phleboviruses 3; CRUV = Candiru virus; CimoPV-IV = Cimo phenuivirus IV; CUMV = Cumuto virus; ECHV = Echarte virus; EWSMV = European wheat striate mosaic virus; FCTenV1 = Fitzroy crossing tenui-like virus 1; GAV = Grand Arbaud virus; GMPV = Guadeloupe mosquito phasivirus; GOUV = Gouléako virus; GORV = Gordil virus; GTV = Guertu virus; HRTV = Heartland bandavirus; HpTV-2 = Huangpi tick virus 2; HbBLV-1 = Hubei bunya-like virus 1; HIGV = Hunter Island Group virus; KAMV = Kabuto mountain virus; KISV = Kismayo virus; LHTV = Lihan Tick Virus; LSV = Lone Star virus; MSpV = Maize stripe tenuivirus; MASV = Massilia virus; MoV = Mourilyan virus; MURV = Murre virus; NWPV1 = Norway phlebovirus 1; PCPhasV1 = Parry's Creek phasivirus 1; PCLV = Phasi Charoen-like phasivirus; PPV = Precarious point virus; RAZV = Razdan virus; RHBV = Rice hoja blanca tenuivirus; RSV = Rice stripe tenuivirus; RVF = Rift Valley fever virus; SAFV = Saint-Floris virus; SFNV = Sandfly fever Naples virus; SFMOV = Sefomo virus; SFTSV = Severe fever with thrombocytopenia syndrome virus; SINV-14 = Solenopsis invicta virus 14; SILV = Silverwater virus; TcTV-2 = Tacheng tick virus 2; TSWV = Tomato spotted wilt orthotospovirus; TOSV = Toscana virus; UUKV = Uukuniemi virus; WzSV1 = Whenzhou shrimp virus 1; YCV = Yichang Insect virus; ZTV = Zaliv Terpenia virus; ZbbV = Zwiesel bat banyangvirus.

Rhabdoviridae

The large viral family *Rhabdoviridae* is home to 40 genera of viruses within the three subfamilies *Alpharhabdovirinae*, *Betarhabdovirinae*, and *Gammarhabdovirinae* [102]. It holds a wide range of viruses that can infect vertebrate and plant hosts, many of which are transmitted by arthropods such as mosquitoes, sandflies, and midges [103-106]. But there are also a number of insect-specific rhabdoviruses, like those in the genus *Almendravirus* (subfamily *Alpharhabdovirinae*) [107-109].

Screening of the mosquito samples for rhabdoviruses yielded sequence fragments of two novel viruses and two previously known viruses. 11 strains of the insect-specific Merida virus (MERDV) (subfamily *Alpharhabdovirinae*, genus *Merhavirus*) were found

and provisionally named Rhabdo II (pairwise amino acid identities of 87.10%–100% to MERDV isolate MERD-Mex07 (KU194360)) [110]. The lower pairwise identity values are primarily due to the low sequence quality of some of the screening fragments. One strain of the mosquito rhabdovirus Cimo rhabdovirus-V (CimoRV-V) (genus unassigned) was found and provisionally named Rhabdo IV (pairwise amino acid identities of 100% to CimoRV-V strain CimoRhV-V/D24-CI-2004 (MZ202305)) [53]. Additionally, we detected two strains of a novel virus, related to Manly virus (MANLV) and CimoRV-V (pairwise amino acid identities of 72.63% - 73.60% to MANLV (MK026564) and 72.13% - 72.63% to CimoRV-V strain CimoRhV-V/D24-CI-2004 (MZ202305)), that was provisionally named Rhabdo I, and six strains of a novel virus related to the mosquito rhabdovirus Cimo rhabdovirus IV (CimoRV-IV) (genus unassigned) (pairwise amino acid identities of 81.57% - 88.09% to CimoRV-IV strain CimoRhV-IV/C68-CI-2004 (MZ202304)), provisionally named Rhabdo III. Species demarcation criteria of the genus *Merhavirus* and *Ohlsrhavirus* were applied for Rhabdo I and Rhabdo III, respectively. For both genera, ICTV proposes a minimum divergence of 10% in L protein amino acid sequence to the next established virus for the definition of a distinct new species [102].

In the phylogenetic tree derived from representative member of each detected viral species and reference sequences derived from GenBank, Rhabdo II closely grouped together with MERDV and Rhabdo IV with CimoRV-V (Figure 10). Rhabdo I clustered basal to the members of the genus *Merhavirus*, and Rhabdo III clustered together with CimoRV-IV, basal to the members of the genus *Ohlsrhavirus*.

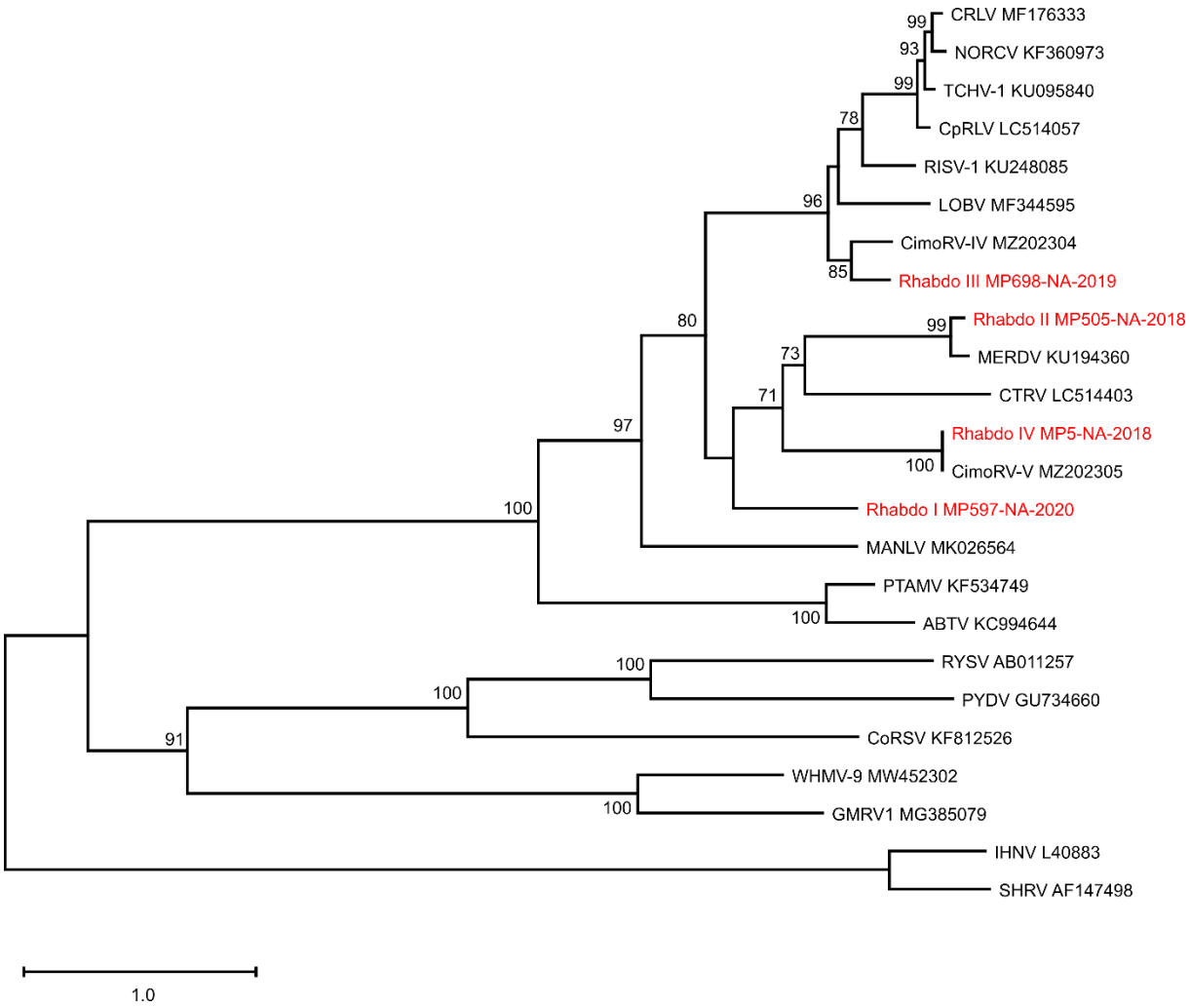

Figure 10: Phylogenetic tree of detected members of the viral genus *Rhabdoviridae*. A maximum likelihood phylogenetic tree was inferred from a partial L protein alignment. The phylogeny was derived with the LG substitution model with a fixed proportion of 0.0 invariable sites, as applied by Smart Model Selection in PhyML (SMS). The phylogenetic tree was midpoint rooted, and support values, based on 1,000 bootstrap iterations, are depicted for each node. Only support values higher than 70% are shown. The viral sequences Rhabdo I–IV from this publication are shown in red. Reference sequences derived from GenBank are shown together with their accession numbers. Abbreviations for reference viruses are as follows: ABTV = Arboretum virus; CimoRV-IV = Cimo rhabdovirus-IV; CimoRV-V = Cimo rhabdovirus-V; CoRSV = Coffee ringspot virus; CpRLV = Culex pseudovishnui rhabdo-like virus; CRLV = Culex rhabdo-like virus; CTRV = Culex tritaeniorhynchus rhabdovirus; GMRV1 = Grenada mosquito rhabdovirus 1; IHNV = Infectious hematopoietic necrosis virus; LOBV = Lobeira virus; MANLV = Manly virus; MERDV = Merida virus; NORCV = North Creek virus; PTAMV = Puerto Almendras virus; PYDV = Potato yellow dwarf virus;

RISV-1 = Riverside virus 1; RYSV = Rice yellow stunt virus; SHRV = Snakehead rhabdovirus; TCHV-1 = Tongilchon virus 1; WHMV-9 = Wuhan Mosquito Virus 9.

Mesoniviridae

The viral family *Mesoniviridae* represents a relatively small group of insect-specific viruses that has only been discovered in the last decade [52, 53, 111-117]. Most mesoniviruses have been found in mosquitoes, though recent publications also report detections in biting midges (family: Ceratopogonidae) and aphids (family: Aphididae) [117, 118].

RT-PCR Screening revealed fragments of two distinct mesonivirus species, six strains of a novel mesonivirus (provisionally designated Mesoni I), related to Hana virus (HanaV) (PEDs of 0.04747–0.07938 in amino acid sequences and pairwise nucleotide identities of 90.45%–92.73% to HanaV strain A4/CI/2004 (JQ957872)) and three new strains of Nam Dinh virus (NDiV) (designated Mesoni II) (pairwise evolutionary distances (PEDs) of 0.00699–0.00703 in amino acid sequences and pairwise nucleotide identities of 96.46%–96.67% to NDiV isolate 02VN178 (DQ458789)). A PED greater than 0.032 in amino acid sequence was applied as the criterion for species demarcation as suggested for the genus *Alphamesonivirus* [114, 119].

In a phylogenetic analysis, Mesoni I grouped with NDiV and its close relative Cavally virus (CavV), while Mesoni II formed a monophyletic clade with HanaV (Figure 11).

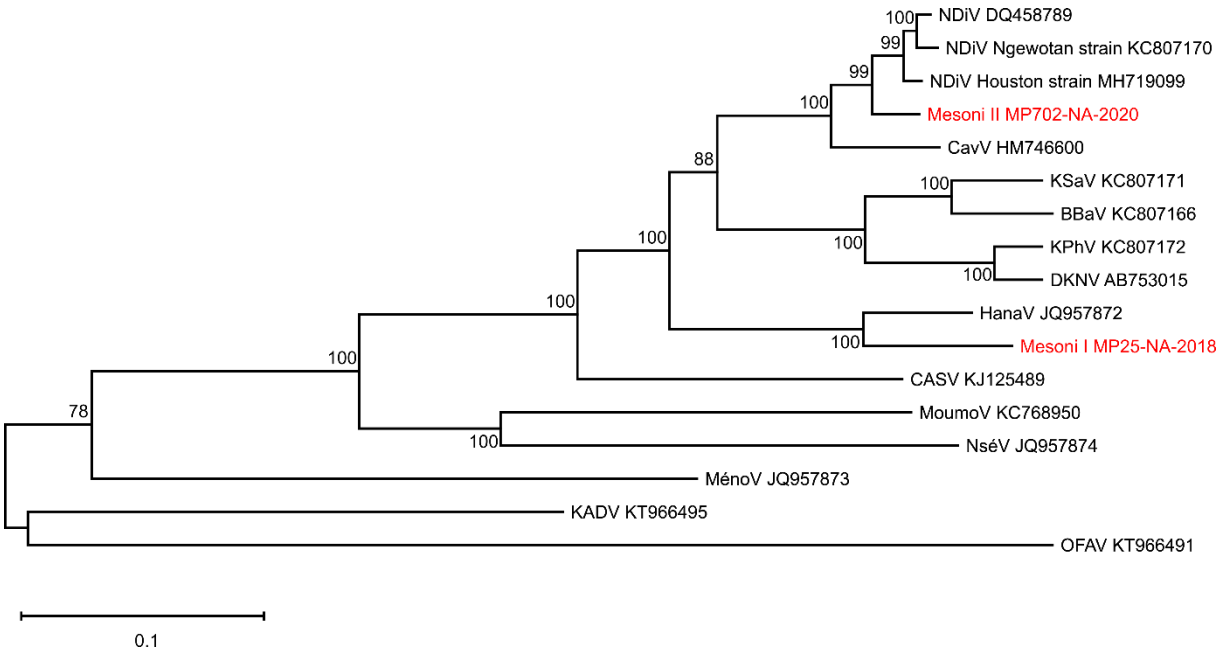

Figure 11: Phylogenetic tree of detected mesoniviruses. A maximum likelihood

phylogeny was derived from a translational nucleotide alignment of the viral ORF1b (encoding the viral RNA-dependent RNA polymerase (RdRP) and other non-structural proteins). The phylogenetic tree was calculated with the Blosum62 model with an estimated gamma shape parameter of 1.27 and an estimated proportion of 0.265 invariable sites, as suggested by Smart Model Selection in PhyML (SMS). The phylogeny was rooted on midpoint, and support values based on 1,000 bootstrap iterations are given for each node. Only support values higher than 70% are shown. Detected viral sequences are shown in red. Accession numbers are supplied for reference sequences, which were all derived from GenBank. The reference viruses were abbreviated as follows: BBaV = Bontag Baru virus; CASV = Casuarina virus; CavV = Cavally virus; DKNV = Dak Nong virus; HanaV = Hana virus; KADV = Kadiwéu virus; KPhV = Kamphaeng Phet virus; KSaV = Karang Sari virus; NDiV = Nam Dinh virus; MénoV = Méno virus; MoumoV = Moumo virus; NséV = Nsé virus; OFAV = Ofaié virus.

Biodiversity and host association of the virus community

The ensemble of detected viruses contained a total of 30 species assigned to 7 different virus families. The virus detections for the different sampling sites are summarized in Table 6. The viral families *Flaviviridae* and *Phenuiviridae* showed the highest species richness, with eleven and nine different virus species, respectively. *Rhabdoviridae*, *Peribunyaviridae*, and *Mesoniviridae* were represented by two to three members each, and *Reoviridae* and *Togaviridae* both contributed a single virus species. Looking at the different sampling sites, the highest virus species richness could be studied in Wuparo Cons. (Figure 12). All sampling sites harbored viruses from two to four viral families, with Wuparo Cons. core area and Wuparo Cons. grazing land exhibiting the highest number of different families.

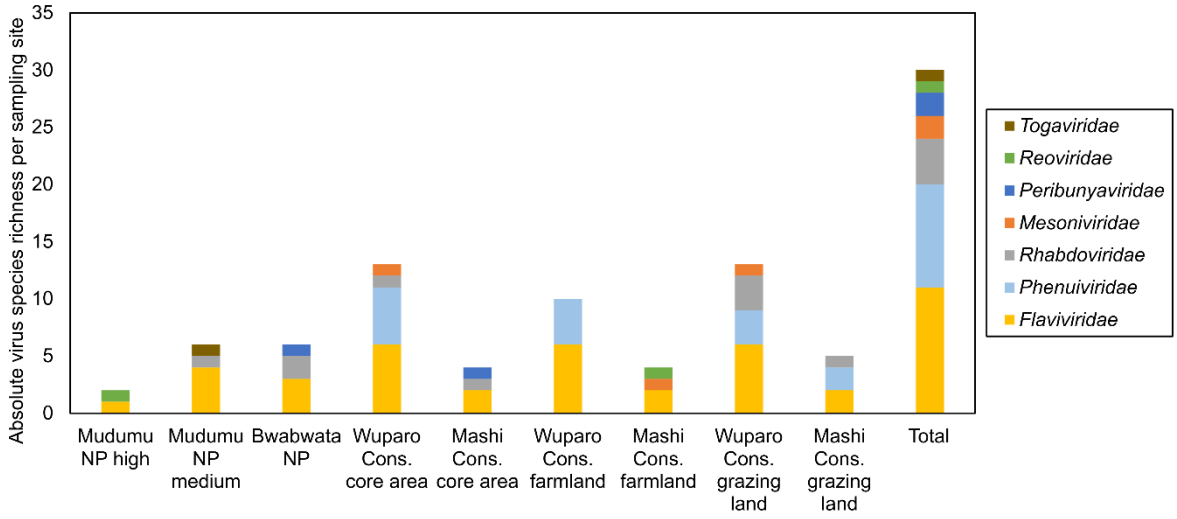

Figure 12: Virus species richness per sampling site, divided by virus families. Total virus species count for sampling sites in National Parks, Conservancy core areas, farmland, and grazing land in the Namibian Zambezi Region.

Table 6: Abundance of detected virus species per sampling site.

	Mudumu NP high	Mudumu NP medium	Bwabwata NP	Wuparo Cons. core area	Mashi Cons. core area	Wuparo Cons. farmland	Mashi Cons. farmland	Wuparo Cons. grazing land	Mashi Cons. grazing land	Total	Total %
Alpha I	0	1	0	0	0	0	0	0	0	1	0.33
Flavi I	1	14	0	5	0	23	0	44	0	87	28.62
Flavi II	0	0	0	0	0	4	0	6	0	10	3.29
Flavi III	0	0	50	1	0	3	3	1	0	58	19.08
Flavi IV	0	1	27	8	0	6	0	0	0	42	13.82
Flavi IX	0	0	0	1	0	0	0	2	0	3	0.99
Flavi V	0	0	0	0	3	4	0	1	2	10	3.29
Flavi VI	0	0	0	1	1	0	0	0	0	2	0.66
Flavi VII	0	0	19	1	0	0	0	0	0	20	6.58
Flavi VIII	0	1	0	0	0	1	0	2	0	4	1.32
Flavi X	0	0	0	0	0	0	7	0	1	8	2.63
Flavi XI	0	3	0	0	0	0	0	0	0	3	0.99
Mesoni I	0	0	0	1	0	0	0	5	0	6	1.97
Mesoni II	0	0	0	0	0	0	3	0	0	3	0.99
Orbi I	1	0	0	0	0	0	2	0	0	3	0.99
Ortho I	0	0	2	0	0	0	0	0	0	2	0.66
Ortho II	0	0	0	0	1	0	0	0	0	1	0.33
Phlebo I	0	0	0	5	0	0	0	1	0	6	1.97
Phlebo II	0	0	0	1	0	2	0	0	1	4	1.32
Phlebo III	0	0	0	0	0	0	0	2	0	2	0.66
Phlebo IV	0	0	0	0	0	1	0	0	0	1	0.33
Phlebo VIII	0	0	0	0	0	1	0	0	0	1	0.33
Phlebo V	0	0	0	1	0	1	0	0	0	2	0.66
Phlebo VI	0	0	0	2	0	0	0	0	0	2	0.66
Phlebo VII	0	0	0	1	0	0	0	1	0	2	0.66
Phlebo IX	0	0	0	0	0	0	0	0	1	1	0.33
Rhabdo I	0	0	0	0	1	0	0	1	0	2	0.66
Rhabdo II	0	1	3	4	0	0	0	1	2	11	3.62
Rhabdo III	0	0	6	0	0	0	0	0	0	6	1.97
Rhabdo IV	0	0	0	0	0	0	0	1	0	1	0.33

Viruses were detected in mosquitoes from the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, and *Mansonia*. Flaviviruses and phenuiviruses were discovered throughout all the aforementioned mosquito genera and rhabdoviruses in all but *Mansonia*. Mesoniviruses were found in *Coquillettidia* and *Culex* mosquitoes. Peribunyaviruses, orbiviruses, and alphaviruses could only be found in mosquitoes of the genus *Culex*. Out of the 30 detected virus species, 13 were specific to a single mosquito species, and the other 17 were found in more than one mosquito species. *Culex univittatus* and *Mansonia uniformis* harbored the highest number of the unique virus species (both three), and *Culex univittatus* gave home to the highest virus species richness (15 distinct virus species from six out of the seven examined virus families). Most of the overall virus detections (85.2%) resulted from the five most abundantly sampled mosquito host species: *Culex univittatus*, *Coquillettidia metallica*, *Mansonia uniformis*, *Coquillettidia fuscopennata*, and *Mansonia africana nigerrima*, which collectively contributed 73.8% of the overall mosquito catch.

Analysis of virus prevalence in the different land-use types

Viruses of the family *Phenuiviridae* were overrepresented in Wuparo Cons. core area, Mashi Cons. grazing land, and Wuparo Cons. farmland (χ^2 = 43.78; degrees of freedom = 8; χ^2_{crit} (α = 0.05) = 15.507; $P \leq 0.001$), *Mesoniviridae* in Mashi Cons. farmland and Wuparo Cons. grazing land (χ^2 = 24.74; degrees of freedom = 8; χ^2_{crit} (α = 0.05) = 15.507; $P \leq 0.005$). *Reoviridae* were significantly more abundant in Mashi Cons. farmland and Mudumu NP high (χ^2 = 74.69; degrees of freedom = 8; χ^2_{crit} (α = 0.05) = 15.507; $P \leq 0.001$). *Peribunyaviridae* were significantly more abundant in Mashi Cons. core area (χ^2 = 17.68; degrees of freedom = 8; χ^2_{crit} (α = 0.05) = 15.507; $P \leq$ 0.05). Most of the detected viruses showed low prevalence levels. Only flaviviruses were found at an exceptionally high frequency. Viruses of the family *Flaviviridae* made up 81% of the total virus detections, while the families *Togaviridae*, *Reoviridae*, and *Peribunyaviridae* each contributed less than 1% of the total virus detections (Table 7).

Table 7: Abundance of sampled viruses, sorted by virus family and sampling site.

	Mudumu NP high		Mudumu NP medium		Bwabwata NP		Wuparo Cons. core area		Mashi Cons. core area		Wuparo Cons. farmland		Mashi Cons. farmland		Wuparo Cons. grazing land		Mashi Cons. grazing land		Total		χ^2-Test	
	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	[N]	[%]	χ^2	P-Value
Togaviridae	0	0.00	1	4.76	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	0.33	13.48	NS
Flaviviridae	1	50.00	19	90.48	96	89.72	17	53.13	4	66.67	41	89.13	10	66.67	56	82.35	3	42.86	247	81.25	6.70	NS
Mesoniviridae	0	0.00	0	0.00	0	0.00	1	3.13	0	0.00	0	0.00	3	20.00	5	7.35	0	0.00	9	2.96	24.74	< 0.005
Reoviridae	1	50.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	13.33	0	0.00	0	0.00	3	0.99	74.69	< 0.001
Peribunyaviridae	0	0.00	0	0.00	2	1.87	0	0.00	1	16.67	0	0.00	0	0.00	0	0.00	0	0.00	3	0.99	17.68	< 0.05
Phenuiviridae	0	0.00	0	0.00	0	0.00	10	31.25	0	0.00	5	10.87	0	0.00	4	5.88	2	28.57	21	6.91	43.78	< 0.001
Rhabdoviridae	0	0.00	1	4.76	9	8.41	4	12.50	1	16.67	0	0.00	0	0.00	3	4.41	2	28.57	20	6.58	13.06	NS

The cumulative minimum infection rate (MIR) was especially high in Bwabwata NP and in Wuparo Cons. grazing land (Figure 13). This is mainly due to the highly prevalent virus species Flavi I, Flavi III, Flavi IV, and Flavi VII (Figure 14), which almost exclusively occurred in the mosquito species *Coquillettidia metallica*, *Mansonia uniformis*, and *Mansonia africana nigerrima*. As a consequence, these mosquito species all showed exceptionally high MIRs: *Mansonia uniformis* (MIR of 67.1 virus detections per 1000 sampled mosquitoes), *Coquillettidia metallica* (MIR of 54.9), and *Mansonia africana nigerrima* (MIR of 94.4). To study the influence of mosquito community composition on the occurrence of virus detections, a subset of data was selected that did not contain these three most dominant mosquito host species. When looking at the MIRs per sampling site for this subset of data, only Wuparo Cons. core area stands out with a higher-than-average MIR and Mudumu NP high with a lower-than-average MIR (Figure 15).

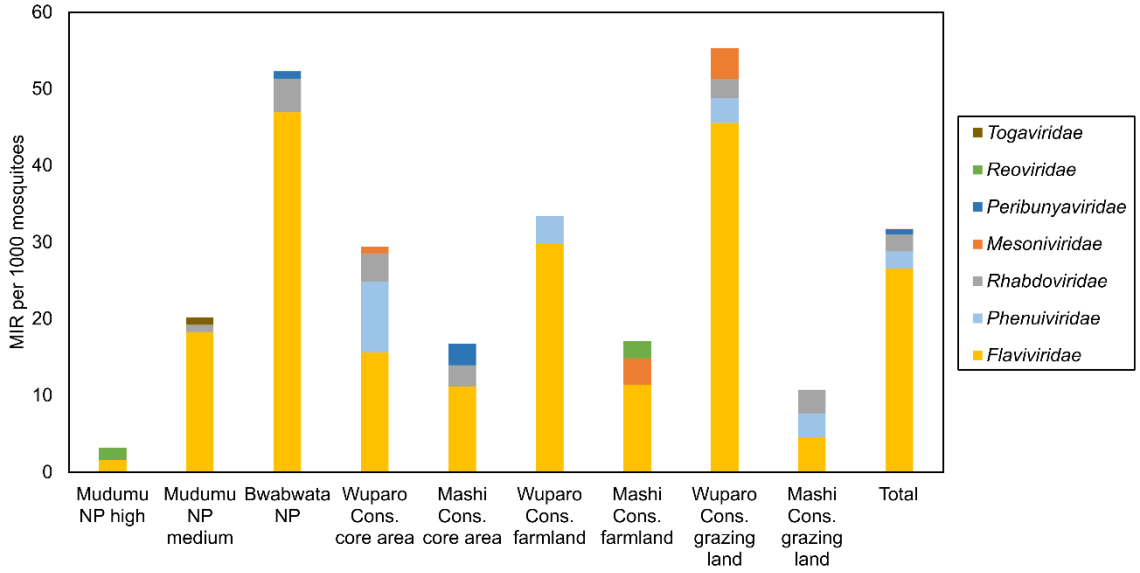

Figure 13: Total MIR per sampling site. Total MIRs were calculated per 1000 individuals for each sampling site and divided by virus families.

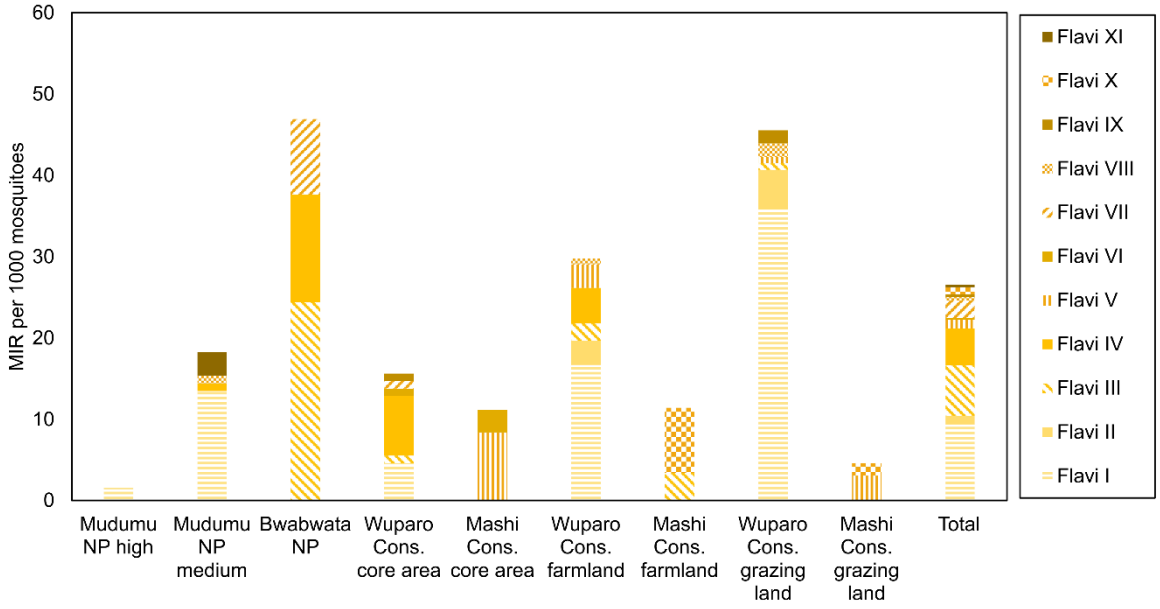

Figure 14: Flavivirus MIRs per sampling site. MIRs were calculated per 1000 individuals for each sampling site for all detected members of the viral family *Flaviviridae*.

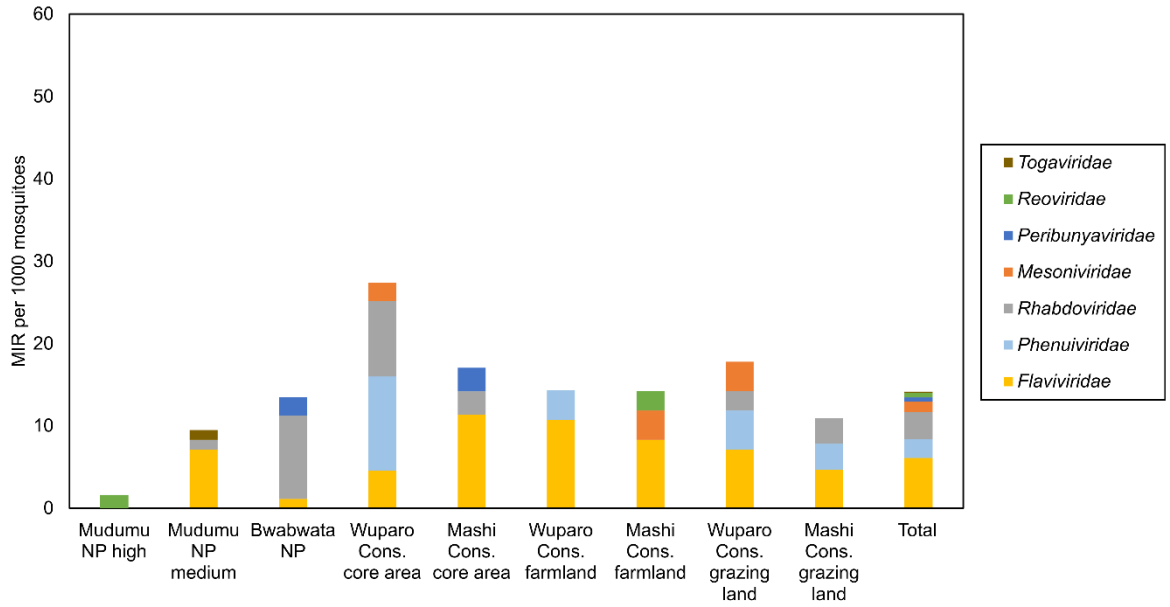

Figure 15: MIRs per sampling site for an adjusted subset of mosquito species. MIRs were calculated per 1000 individuals for each sampling site for a subset of data that did not contain the three most dominant mosquito host species.

The detected virus species were further examined by NMDS ordination analysis (Figure 16). Altogether, 7 out of 31 detected virus species significantly correlated with the ordination axes, which explained 70% (Axis 1) and 11% (Axis 2) of the total variation (NMDS stress value = 0.06185) (Table 8). The NMDS analysis of mosquito virus community composition for the different land-use types showed a similar distribution to the NMDS analysis of mosquito and mosquito vector community compositions. Bwabwata NP is placed on the far-right side of the diagram, Wuparo Cons. sampling sites cluster in the bottom left corner, and Mashi Cons. sampling sites are in the top left corner. Mudumu NP sampling sites are placed between Wuparo and Mashi Cons..

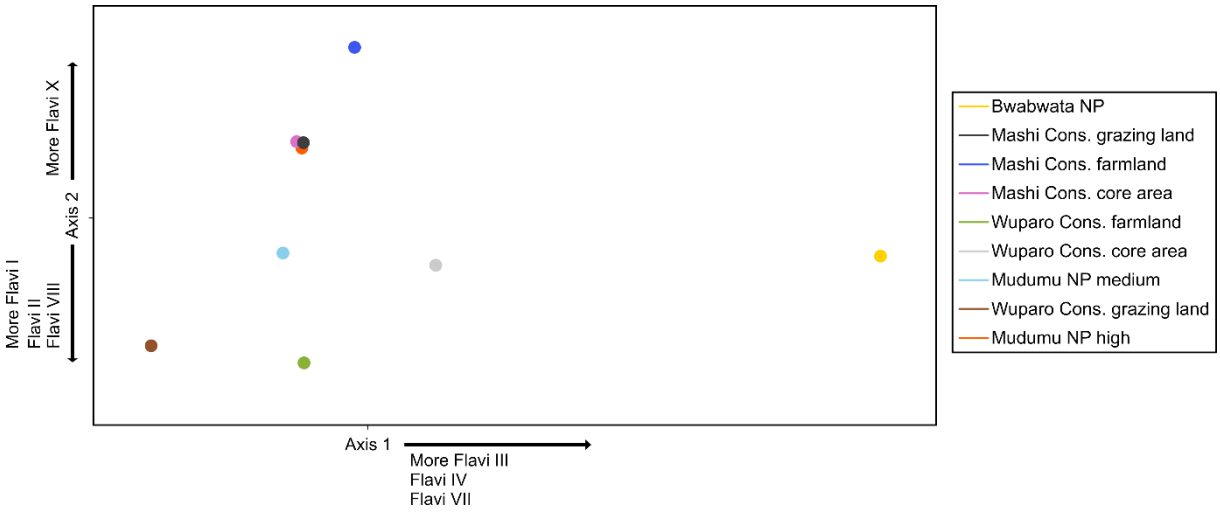

Figure 16: Ordination analysis of the virus community composition. Axis 1 of the NMDS analysis showed clear differences between Bwabwata NP on the right side of the graph and the rest of the sampling sites. Wuparo Cons. sampling sites in the bottom part of the diagram were separated by Axis 2 from Mashi Cons. sampling sites in the top part of the diagram.

Table 8: Pearson correlation coefficients with two NMDS ordination axes of the significantly correlated virus species. The correlation coefficients that were highly significant ($P \leq 0.001$) using a Monte Carlo permutation test and a Bonferroni correction are shown in bold type.

	Axis 1 coefficient	P-value	Axis 2 coefficient	P-value
Flavi I	-0.42134	0.2626	**-0.84853**	**0.0046**
Flavi II	-0.3749	0.2886	**-0.76226**	**0.0141**
Flavi III	**0.85979**	**0.0399**	-0.17863	0.6612
Flavi IV	**0.82391**	**0.0094**	-0.44153	0.2233
Flavi VII	**0.97649**	**0.0104**	-0.14078	0.7948
Flavi VIII	-0.44976	0.1769	**-0.75361**	**0.0284**
Flavi X	-0.10489	0.8712	**0.67246**	**0.041**

Discussion

A diverse set of mosquito and virus communities was described from mosquito samples originating in different locations in National Parks and Conservancies in the Namibian Zambezi Region. The total ensemble presented a high mosquito species richness of 33 species from 9 genera of Culicidae and a similarly high virus species richness of 30 species of RNA viruses from the seven most common arthropod-borne virus (arbovirus) and insect-specific virus families (*Flaviviridae*, *Mesoniviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae*). 19 of

the 30 detected viruses proposedly resemble novel species: 17 putative insect-specific viruses (ISVs) and two potential arboviruses.

Based on our replicated samples, Mashi Cons. sampling sites had slightly fewer mosquito captures than Wuparo Cons. sampling sites, and Mudumu NP had fewer than Bwabwata NP. Despite the uneven distribution of catch sizes, we did not further standardize our sample data set as we were using equal sampling intensity for each site (equal-effort sampling). As a general problem of equal-effort sampling, biodiversity indices derived for communities with fewer sampled specimens tend to be underestimated [120]. The coverage of the sampling was evaluated with a species accumulation curve. When the sampling coverage is coming close to its endpoint, the species accumulation curve approximates its asymptote [121]. All species accumulation curves suggested an appropriate sampling effort, though more trapping nights might have added a few more very rare species.

Chi-square testing of the different sampling sites showed an uneven distribution of mosquito subfamilies, tribes, and sexes, which gave a first indication of differences in the mosquito community compositions. The distribution of species abundances in the different sampling sites was examined for a better understanding of the mosquito community composition [26, 27]. Rank-abundance analysis portrayed mosquito communities as dominated by a few very common species, with a lengthy tail of infrequent species. *Culex univittatus* and *Mansonia uniformis*, which could be found in all sampling sites, were identified as the two most generalist species of the sampling set.

For the entire ensemble, high biodiversity indicators could be observed, considering that all samples were collected from areas with very restricted aquatic breeding grounds for larvae. The comparison of mosquito species richness and Shannon diversity index revealed significant differences between the two sampled National Parks and Conservancies, respectively. However, sampling sites within each of the Conservancies showed consistent species richness and, only for Wuparo Conservancy, a differently shaped Shannon index. Species richness is one of the most basic and important indicators of the biodiversity of a biological community. It is a very simple concept, only taking into account the absolute number of individual species in an ecological system [18, 23, 24]. Among the other indicators in use for indexing biological diversity, the Shannon (or Shannon-Wiener) and Simpson indices are among the most common ones [122]. In comparison to the Shannon index, Simpson

diversity (represented by the letter "λ") is defined by the sum of individual species proportions raised to the second power ($\lambda = \sum_{i=1}^{n} pi^2$) [123]. However, by the makeup of their mathematical formulas, both indices should give correspondent biodiversity rankings as long as the populations that are examined lie within the same distribution ranges [122].

Ordination analysis showed that mosquito community composition differed greatly depending on the sampling site. Wuparo and Mashi Cons. mosquito communities were clearly separated from each other, with Mudumu NP sampling sites clustering approximately in the middle of them. Mashi Cons. sampling sites showed a big overlap of mosquito communities, and the biggest differences were found between Bwabwata NP and all other sites. The ordination analysis of the mosquito disease vector community showed about the same pattern as the ordination analysis of the entire mosquito community. Of note, in Bwabwata NP, more known disease vector species were observed than in all other sampling sites, and in Mashi Cons., slightly more than in Wuparo Cons. sampling sites.

The total virus ensemble yielded five arboviruses, 24 ISVs, and one potential plant arbovirus. Two of the arboviruses are well-studied confirmed arboviruses, one is a potential known arbovirus, and two are potential novel arbovirus species that were classified due to their phylogenetic grouping. Peribunya II grouped together with two potential arboviruses, KOOV and WONV. Both of them were originally isolated from *Culex annulirostris* mosquitoes, sampled in Queensland, Australia [124]. There is serological evidence (for KOOV HI and NT; for WONV HI) that KOOV and WONV can infect a wide range of vertebrates, including humans, and they were both isolated via intracerebral infection of mice [124-126]. However, they have both not been associated with any disease so far. Peribunya I grouped together with HSIV-1, a virus that has been isolated from *Sesamia inferens* and *Chilo suppressalis* moths – two species that are known for being notorious crop pests – collected in Hangzhou, China [127, 128]. So far, only sequence and sampling information for HSIV-1 (NCBI:txid2905550) has been published. In phylogenetic analysis HSIV-1 and Peribunya I are forming a distinct new clade of monophyletic origin that is basal to the clade formed by the members of the peribunyaviral genus *Herbevirus*. Therefore, HSIV-1 and Peribunya I are proposed as members of a new genus within the viral family *Peribunyaviridae*, tentatively named *Hangzhovirus*.

Of the ISVs, seven resemble detections of previously known virus species, and 17 are putatively novel ISVs, which were classified by their phylogenetic position. In phylogenetic analysis, the novel phenuiviruses Phenui III, Phenui VII, Phenui VIII, and Phenui IX grouped within the well-defined clade formed by the established phasiviruses PCLV, BADUV, and GMPV. Phenui III, Phenui VII, Phenui VIII, and Phenui IX are therefore proposed as new members of the phenuiviral genus *Phasivirus*. Phenui II and Phenui VI clustered within the group around GOUV, YCV, and CUMV and are proposed as new members of the *Goukovirus*. Phenui IV's genus affiliation could not be determined conclusively by phylogenetic grouping because it was placed in the basal position to the clade of unassigned viruses surrounding NWPV1, BLTPV1, and BLTPV3. Several genetically divergent strains of the proposed *Tenuivirus* FCTenV1 (originally detected in *Culex annulirostris* from Kimberley, Australia) were found [97]. Although the full host tropism of FCTenV1 remains to be elucidated, its phylogenetic grouping suggests its affiliation with the phenuiviral genus *Tenuivirus*, a group of economically important plant viruses that are primarily transmitted by leafhoppers of the family Delphacidae [97, 129, 130].

The two novel rhabdoviruses that were detected could not clearly be assigned to established genera, and for the moment they will be defined as unclassified members of the family *Rhabdoviridae*. In phylogenetic analysis, Rhabdo I was placed basal to the members of the genus *Merhavirus*, and Rhabdo III grouped together with CimoRV-IV (genus unassigned) in basal position to the members of the genus *Ohlsrhavirus* [53]. Mesoni I formed a monophyletic clade with HanaV within the group of alphamesoniviruses, and its amino acid sequence showed a PED greater than 0.032 to HanaV. Therefore, Mesoni I is proposed as a new member of the genus *Alphamesonivirus* within the family *Mesoniviridae*.

For further classification of the 19 putatively novel virus species, more extensive sequence analysis and, if they can be obtained, the characterization of live virus isolates, will be necessary.

Wuparo Cons. appeared to have a particularly high diversity of virus species. However, this assertion should be considered with caution as the trap count of our mosquito sampling prevented the generation of virus community replicates. It is a general disadvantage of the species richness concept that a very large sample size is necessary when researching microorganisms like viruses that are dependent on a host

animal for their replication [131]. As an alternative, a statistical estimate of the total species richness count could be made [132].

The five mosquito species *Culex univittatus*, *Mansonia uniformis*, *Coquillettidia metallica*, *Coquillettidia fuscopennata*, and *Mansonia africana nigerrima* were identified as the dominant host species in the sample set. All five of them are known as notorious vectors of zoonotic viruses and can be found on the WRBU list of medically important mosquito species (http://www.wrbu.org/mqID/keysMQAlpha.html).

Virus prevalence as measured by MIR was especially high in Bwabwata NP and Wuparo Cons. grazing land. However, if the most dominant mosquito host species are not considered for the calculation of MIRs, it can be seen that virus infection rates are strongly influenced by the presence and abundance of certain key mosquito species. This phenomenon has been coined the “abundance effect” by Hermanns et al. [53]. The ordination analysis of virus community composition in dependence on sampling sites resembled the ordination analysis of mosquito host community composition, which indicates a strong correlation between these two communities.

In the analysis of our data, no clear correlation between mosquito host biodiversity and virus prevalence could be deduced. On the one hand, the comparison between Bwabwata and Mudumu NP showed higher host biodiversity, supporting higher viral infection rates. In the comparison of Wuparo and Mashi Cons., on the other hand, higher host biodiversity resulted in lower viral infection rates. This is in line with previous findings that suggest that the implications of increased host biodiversity are context-dependent and that increased biodiversity can have both a diluting and amplifying effect on the prevalence of infectious disease [133-137]. In some cases, both effects are at work at the same time and compete for an overall impact (amplification, dilution, or no net effect) [137]. In general, the prevalence of vector-transmitted disease is thought to be subject to the dilution effect, but additional factors, like host preferences and vector competence, can have a strong additional influence [134, 138-141]. Others argue that the association between total biodiversity and disease prevalence is only of minor significance and that factors like the host community composition are more important for the prevalence of infectious disease than biodiversity [142-144]. For the dataset at hand, mosquito community composition seemed to play a more important role in the occurrence of virus infections than total biodiversity. In the rank abundance analysis that was performed for the sampled mosquitoes, it became clear that all mosquito host communities were dominated by a

few very abundant species. All the other species sampled occurred in very small numbers. They carried a lot of weight in the calculation of biodiversity indices but only marginally contributed to the total occurrence of virus infections. Furthermore, comparing the ordination analyses of mosquito and virus communities in the different sampling sites gave another indication that mosquito community composition influenced the complexion of observed virus communities significantly.

Virus transmission will most likely be mediated by one of the mosquito vector species, which is most commonly and abundantly found and shows the highest infection rates. This especially highlights *Culex univittatus* and *Mansonia uniformis*, which should both be regarded with special attention in future studies.

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Conflict of interest

The authors have declared that no competing interests exist.

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General Discussion

Mosquito-borne arboviruses of the Namibian Zambezi region

Mosquitoes are well-known vectors for a large number of arboviruses that can infect vertebrate hosts, ranging from amphibious frogs to humans [1-4]. Several strains of three known arboviruses and two putative novel arboviruses from the viral families *Flaviviridae*, *Reoviridae*, and *Peribunyaviridae* were discovered in the mosquitoes that were sampled and examined for this study.

Ten genetically diverse strains of the human pathogenic West Nile virus (WNV) have been identified in the viral family *Flaviviridae*. WNV is an emerging and reemerging infectious disease of global importance that has also spread in large parts of the Americas and Europe in recent decades [5-8]. WNV shows a high level of genetic diversity, which phylogenetically groups into five to nine lineages with several subclades [9-11]. WNV lineage 1 in particular has been linked to disease in birds and mammals, but recent findings suggest that lineage 2 also harbors a variety of highly neuroinvasive strains, and pathogenicity may not be linked to lineage after all [12-20]. The detected WNV strains all grouped into two different clades of WNV lineage 2, which is the predominant WNV lineage in southern Africa [19]. Unfortunately, no virus isolate could be obtained from the detected WNV strains due to an accidental interruption of the cold chain during the transport of the respective mosquito samples. Therefore, it was not possible to assess their pathogenicity in infection experiments. However, each of the strains probably harbors the potential to be a highly neuroinvasive type of the virus. Therefore, additional mosquito samplings are advised to obtain live isolates and assess the possible risk emanating from the WNV strains circulating in the region. Furthermore, for a better estimation of overall WNV infection events, serological sampling of the local population and mosquito monitoring will be necessary.

Additionally, we detected and isolated three strains of Bagaza virus (BAGV), which is another arboviral member of the *Flaviviridae* virus family. BAGV is a well-known avian pathogen that caused regular outbreaks with high mortality rates in Israeli turkey farms until a vaccine was developed to combat it (the virus is also known under the names Israel turkey meningoencephalomyelitis virus (ITV) and Avian meningoencephalomyelitis virus (AMEV)) [21-24]. Apart from turkeys, the virus strongly affects other phasianid birds, such as partridges and pheasants, that were

found infected in Spain [25], South Africa [26], and Portugal [27]. Serological data (neutralizing antibodies) from hospitalized encephalitis patients in India also suggests the infection of humans; however, it is not yet clear if BAGV causes disease symptoms [24, 28]. In cell culture infection experiments with one of the isolated BAGV strains from Namibia, high replication rates could be observed in the avian cell line AGE1.CR (derived from Muscovy duck) and significantly lower replication rates in mammalian cell lines derived from humans (HEK293T), primates (VeroE6), and rodents (BHK-21). This corresponds with the proposed enzootic transmission cycle between birds and ornithophilic mosquitoes, with mammals as incidental dead-end hosts [28]. Several other tested avian and mammalian cell lines (DF-1 and LMH, both derived from domesticated chicken; and KNR, derived from cattle) did not support the replication of the BAGV isolate from Namibia. Furthermore, BAGV has only been found in a limited number of bird species. So far, there is only one report of a single detection outside of the Phasianidae family [27]. Therefore, a rather narrow host range of specific bird species is suspected. In order to evaluate the effects of a potential BAGV infection on the endemic bird species of the Namibian Zambezi Region, it will be necessary to monitor the local bird population. As a basic solution, this could be done by the collection of deceased wild birds or, more elaborately, by trapping wild birds. Investigation of the local bird population could also bring further insights into the transmission dynamics of WNV, which is also maintained in a mosquito-bird cycle [29, 30].

In the viral family *Reoviridae*, we found three strains of the potential arbovirus Corriparta virus (CORV). CORV has previously been detected in birds and mosquitoes from various parts of the world, but so far, it remains unclear if it induces any disease symptoms in vertebrates [31-34]. CORV has been reported to be pathogenic for intracerebrally injected infant mice and to induce a strong cytopathic effect (CPE) in BHK-21 (derived from rodent) and PS and PK-2a cells (both derived from pig) [31, 34, 35]. One of the Namibian CORV strains detected in this study could be isolated and tested in cell culture infection experiments. The virus replicated at low levels in AGE1.CR cells (derived from Muscovy duck), but no replication was detected in the tested mammalian cell lines (HEK293T, derived from human; VeroE6, derived from African green monkey; and BHK-21, derived from hamster). As a result, the question arises as to whether the Namibian CORV variants exhibit different host tropism than the original CORV isolates, and if so, what the determinants and mechanisms for this

altered host spectrum could be. The recently detected Parry's Lagoon virus (PLV) shows a similar genetic distance to CORV, like the detected Namibian CORV strains [36]. Because of its inability to replicate in vertebrate cell lines (DF-1, derived from domesticated chicken; VeroE6, derived from African green monkey; and BHK-21, derived from hamster) the virus has been proposed as a novel species within the genus *Orbivirus* [36]. If PLV is indeed considered a distinct virus species, the Namibian CORV strains should also be grouped into this novel species, as they share the highest genetic identities and similar growth characteristics. PLV and the Namibian CORV strains might represent intermediate stages in virus evolution that link the transition of an insect-restricted virus to a genuine arbovirus or vice versa.

In the viral family *Peribunyaviridae*, two tentative novel arboviruses were found. Peribunya II is phylogenetically grouped with Koongol virus (KOOV) and Wongal virus (WONV), two potential arboviruses that were originally isolated from mosquitoes in northern Australia [31]. KOOV induced pathogenicity in intracerebrally injected infant mice, and KOOV neutralizing antibodies were found in the sera of several vertebrates, including humans, wallabies, and domestic fowl [31, 37]. Umbre virus (UMBV) of the closely related Turlock serogroup has just recently been identified as the cause of fatal encephalitis in two immunocompromised humans [38]. Unfortunately, no isolate of Peribunya II could be obtained due to an accidental interruption in the cold chain when transporting the respective mosquito samples. Consequently, the virus could not be used in cell culture infection experiments. Host tropism and pathogenicity of the putative novel virus will remain elusive until a new strain of the virus can be found and isolated through further mosquito sampling. Furthermore, only a small portion of the viral genome of Peribunya II has been sequenced thus far, and more genome information will be required for a comprehensive molecular and phylogenetic characterization of the virus.

Peribunya I is a potential novel arbovirus that showed the closest genetic similarities with the unclassified peribunyavirus Hangzhou sesamia inferens peribunyavirus 1 (HSIV-1) found in two different types of crop pest moths in western China. HSIV-1 and Peribunya I formed a new clade of peribunyaviruses, phylogenetically embedded in basal position to the members of the insect-specific genus *Herbevirus* and in apical position to the insect-specific genus *Shangavirus*. Unfortunately, no virus isolate was obtained from the two detected strains of Peribunya I, so the putative host tropism of

the novel virus could not be examined in cell culture infection experiments. Due to their phylogenetic position, HSIV-1 and Peribunya I could be suspected as members of a novel genus of insect-specific peribunyaviruses. This is, however, only a suspicion, and until the potential host tropism can be elucidated with the help of a Peribunya I live virus isolate, the newfound species will remain a potential new arbovirus, like most other members of the *Peribunyaviridae* viral family [39].

Infection rates with arboviruses varied greatly between virus families and species encountered. Members of the viral family *Flaviviridae* were encountered the most frequently, followed by members of the family *Reoviridae*, and members of the family *Peribunyaviridae* were encountered the least frequently. The families *Phenuiviridae*, *Rhabdoviridae*, and *Togaviridae* yielded no arboviral encounters at all. With an approximate infection prevalence of 0.1%, WNV showed the highest infection rate, while all other detected arboviruses were found at frequencies of less than 0.03%. In southern Africa, WNV infection rates in mosquitoes between 0.026% and 3.9% have been found in other studies, while the latter one relates to an epizootic outbreak of WNV [40]. Therefore, the observed WNV infection rate does probably not correspond to a full-scale WNV outbreak but might represent a state of elevated WNV prevalence with epizootic potential.

Mosquito-specific viruses of the Namibian Zambezi region

The subdivision of insect-specific viruses that is associated with mosquitoes is called mosquito-specific viruses (MSVs) [41]. Most mosquito-specific viruses that are known to date have only been established in the last decade [42]. Except for *Reoviridae* and *Peribunyaviridae*, all viral families studied harbored MSVs, which were assigned to seven previously known viruses and 17 potential novel virus species.

The families *Flaviviridae* and *Phenuiviridae* showed the highest diversity of MSVs, with nine and eight distinct species detected, respectively. *Rhabdoviridae* contributed four and *Mesoniviridae* two distinct MSV species. In the viral family *Togaviridae*, only one single strain of the well-studied MSV Eilat virus (EILV) was found. This is in line with previous studies that proved an extensive genetic diversity in the arthropod-specific viruses of the viral families *Flaviviridae*, *Phenuiviridae*, *Rhabdoviridae*, and *Mesoniviridae* [42, 43]. No MSVs were detected in the viral family *Reoviridae*, although there are several examples of insect-specific reoviruses [42]. Maybe this could point out that the RT-PCR assay that was used for the detection of reoviruses should be re-

evaluated. In addition to the detected MSVs, in the *Phenuiviridae* viral family, we found several strains of the proposed *Tenuivirus* Fitzroy crossing tenui-like virus 1 (FCTenV1). The host-tropism of this putative novel virus is still unclear, but its phylogenetic grouping suggests an association with leafhopper-transmitted plant viruses [44].

A full genome sequence and a live virus isolate were derived to investigate the Namibian EILV variant, EILV-MP458-NA-2018. All other detected MSVs have so far only been characterized based on 124–522 nt short RT-PCR screening fragments, and no virus isolation attempts have been made. In order to better assess the putative host tropism of all these novel putative MSV species, live virus isolates will have to be derived and studied in infection experiments. This is especially true for virus species with unclear and poorly supported phylogenetic grouping. For example, the putative novel virus Phenui IV was placed in basal position the genus *Phlebovirus*, adjacent to Blacklegged tick phlebovirus 1, Blacklegged tick phlebovirus 3, and Norway phlebovirus 1 [45-47]. Phenui IV may therefore represent a new member of the arboviral genus *Phlebovirus*, but a very low bootstrap support value at the respective nodes makes a clear classification very difficult.

Infection rates varied greatly between the detected MSV species. The highest infection rates were found in the viral family *Flaviviridae*. Flavi I showed an infection prevalence of 0.9% (87/10,206 mosquitoes), Flavi III showed an infection prevalence of 0.6% (60/10,206 mosquitoes), and Flavi IV showed an infection prevalence of 0.4% (43/10,206 mosquitoes). The members of all other viral families were detected at substantially lower infection rates of 0.01% (1/10,206 mosquitoes) to 0.1% (12/10,206 mosquitoes). This corresponds to previous findings that have shown that insect-specific flaviviruses (ISFs) in mosquito populations are often found at high prevalence levels [48-50], while other groups of insect-specific viruses are mostly found at very low prevalence levels, which is especially the case for the insect-specific alphaviruses [51-55].

It has been observed that pre-existing infections with MSV can influence the replication, transmission, or dissemination of co-infecting or superinfecting arboviruses [56-61]. This has most prominently been shown within the viral family *Flaviviridae*, where interactions between MSVs and arboviruses are well documented [56, 58, 62-67]. Among the studied flaviviruses is the MSV species Culex flavivirus (CxFV), which

was also encountered in ten of our mosquito pools. Experiments with different CxFV strains have shown either a repressing [64, 67] or amplifying [62, 63] effect on co-infecting or superinfecting WNV strains. If it is possible to obtain a virus isolate of one of the Namibian CxFV strains, it will be interesting to check its effects on superinfecting WNV and BAGV. In the viral family *Togaviridae*, EILV was shown to interfere with subsequent Chikungunya virus (CHIKV), Sindbis virus (SINV), eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis (VEEV) infections [57]. In addition to the replicational reduction of superinfecting CHIKV and SINV in cell culture experiments, the novel EILV strain from Namibia was also shown to reduce the replication rates of the flaviviruses WNV and BAGV. This demonstrates the interaction of mosquito viruses across a wide taxonomic range. Only a very few of the virus-positive mosquito pools were infected with multiple virus species (6 out of 315 virus-positive mosquito pools in total), indicating a very low probability of co-infections. However, some co-infections could have been overlooked as the limitations of RT-PCR screening techniques make it very difficult to detect multiple viruses from the same family in a single mosquito pool.

Mosquito community composition in the Namibian Zambezi Region and its effect on associated viruses

The analysis of the sampled mosquito communities and their associated viruses showed strong variations. Both mosquito biodiversity and the general mosquito community composition of the sampled Bwabwata and Mudumu National Parks and Mashi and Wuparo Conservancies varied greatly. Only sampling sites within the two Conservancies showed considerable similarities in their overall biodiversity. However, all the sampled mosquito communities were dominated by a small number of very abundant species, while the majority of encountered species were only found in very low numbers. A comparison of the mosquito communities in Bwabwata and Mudumu National Parks showed considerable variation in their biodiversity and community composition. Similar findings were observed when comparing the sampling sites in Mashi and Wuparo Conservancies.

The biodiversity of mosquito communities and the infection prevalence of associated viruses could not be conclusively correlated with each other in this study. When comparing Bwabwata and Mudumu National Parks, increasing infection rates were

observed in sampling sites with increasing mosquito biodiversity, which is in agreement with an amplification effect [68, 69]. However, the comparison of Mashi and Wuparo Conservancies showed decreasing infection rates in sampling sites with an increasing mosquito species diversity, which is in agreement with a dilution effect [69-72]. This undirected effect of biodiversity on infection prevalence has previously been reported for both mosquito and tick vector species communities [73-76].

However, virus community composition was found to be highly dependent on mosquito community composition, and was primarily shaped by key mosquito species that were responsible for the majority of virus detections.

Mosquito species that are of particular relevance either harbor very high virus diversities, which suggests a higher likelihood that they also include zoonotic or potentially zoonotic viruses [77, 78], or they exhibit particularly high infection rates, increasing the likelihood of coming into contact with an infected individual [79, 80]. Regarding our study, this perspective puts the main focus on the mosquito species *Culex univittatus* and *Mansonia uniformis*, which were identified as the main drivers of virus infections. *Culex univittatus* harbored the biggest biodiversity of viruses, with a total of 15 different viruses, including three (potential) arboviruses, among them the human-pathogenic WNV and avian-pathogenic BAGV. Additionally, *Culex univittatus* is known to host other important arboviruses like Sindbis and Usutu viruses [81-84]. *Mansonia uniformis* showed particularly high infection rates. (MIRs of 35.8 and 23.7 virus detections per 1000 sampled mosquitoes in the top two MSVs). Although we only found MSVs in *Mansonia uniformis*, it is a well-known disease vector for arboviruses like Rift Valley fever virus and Ndumu virus [85, 86]. Both mosquito species will therefore be of special interest in upcoming studies, and it will be of future use to determine the factors that influence their amplification.

Conclusion

An ensemble of mosquito specimens was sampled from four different land-use types in National Parks and Conservancies in the Namibian Zambezi Region. A diverse set of mosquito communities, varying in composition and assessed biodiversity, was described for all sampling sites. The examined mosquitoes harbored a high genetic diversity of RNA viruses across seven common arboviral and insect-specific virus

families. Most importantly, the well-known arboviruses WNV and BAGV and the potential arbovirus CORV were identified among the virus detections. Cell culture experiments could be used to examine the growth kinetics and potential host tropism of the BAGV and CORV virus isolates. Furthermore, a high number of putatively novel viruses was detected, most of which represented MSVs from the viral families Flaviviridae and Phenuiviridae. Infection prevalence and virus community composition in the different sampling sites were studied with regard to varying land-use types and mosquito communities. No conclusive correlation could be derived between mosquito host biodiversity and RNA virus infection prevalence. The composition of the mosquito community, however, was found to have a significant impact on the infection prevalence of the viruses studied.

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List of publications

1. Guggemos HD, Fendt M, Hieke C, Heyde V, Mfune JKE, Borgemeister C, Junglen S. Simultaneous circulation of two West Nile virus lineage 2 clades and Bagaza virus in the Zambezi region, Namibia. PLoS Negl Trop Dis. 2021 Apr 2;15(4):e0009311. doi: https://doi.org/10.1371/journal.pntd.0009311. PMID: 33798192; PMCID: PMC8046352.

2. Guggemos HD, Fendt M, Hermanns K, Hieke C, Heyde V, Mfune JKE, Borgemeister C, Junglen S. Orbiviruses in biting midges and mosquitoes from the Zambezi region, Namibia. J Gen Virol. 2021 Sep;102(9). doi: https://doi.org/10.1099/jgv.0.001662. PMID: 34554079.