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Virome characterization and identification of a putative parvovirus and poxvirus in bat ectoparasites of Yunnan Province, China

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ABSTRACT

Ectoparasites found on bats are known to contain important microbes. However, the viruses hosted by these obligate parasites are understudied. This has led to the near oversight of the potential role of these ectoparasites in virus maintenance and transmission from bats to other interacting species and the environment. Here, we sampled bat ectoparasites parasitizing a diverse selection of bat species in the families Rhinolophidae, Vespertilionidae, Megadermatidae, Hipposideridae and Pteropodidae in Yunnan Province, China. We show that the ectoparasite prevalence was generally higher in male compared to female bats. Most ectoparasites were found to fall within the Nycteribiidae, Spinturnicidae and Streblidae bat ectoparasite families. We subsequently applied a non-biased sequencing of libraries prepared from the pooled ectoparasites, followed by an *in-silico* virus-centric analysis of the resultant reads. We show that ectoparasites hosted by the sampled families of bats are found to carry, in addition to a diverse set of phages, vertebrate and insect viruses in the families *Aliusviridae, Ascoviridae, Chuviridae, Circoviridae, Flaviviridae, Hepadnaviridae, Hepeviridae, Herpesviridae, Iridoviridae, Marseilleviridae, Nairoviridae, Orthomyxoviridae, Parvoviridae, Poxviridae, Reoviridae, Retroviridae, and Rhabdoviridae*. We further report a partial Parvovirus VP1/VP2 gene and partial Poxvirus ubiquitin-like gene predicted by two independent next generation sequencing data analysis pipelines. This study describes the natural virome of bat ectoparasites, providing a platform for understanding the role these ectoparasites play in the maintenance and spread of viruses to other animals.

1. Introduction

Arthropods are among the most abundant animals, making up the majority of global animal biomass [1,2]. Owing to their ubiquity along with their size, arthropods (particularly those that commute between hosts) are exemplars of hyper-active ectoparasitism. This is expected, since most other animals are relatively much larger in size, with parasite sizes being positively correlated with host sizes (*Harrison's rule*) [3].

The role of ectoparasites in transferring microbes or pathogens between their hosts is poorly understood. Mammals host diverse parasite species, in which the community of infesting arthropod species may vary depending on host specificity, host immunity and interspecific interactions. This means that the number and diversity of parasites on individual hosts will vary across space and time [4]. Most of these

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interactions are transient but can be heavily consequential. A case in point is the transmission of *Plasmodium* (Haemosporida), the causative agent for malaria, by free living Anophelinae mosquitoes [5,6]. In this well-studied vector system, mosquitoes maintain the plasmodium parasite in human communities by mediating transmission to naïve hosts. In their sylvatic circulations, mosquitoes on the other hand transmit arthropod-borne viruses including Dengue and Chikungunya viruses from non-human primates to humans [7]. These phenomena underline the interdependence between human health and the health of wild animals in the presence of competent pathogen vectors. These not only provide a large surface area for arthropod habitation, but also the acquisition of pathogens shuttled by the arthropods from diseased animals in their environments. Additionally, ectoparasite load may be associated with individual health, as well as directly impacting on health, and thus the presence of parasites could be an indicator of health or stress, and then if capable of transmitting pathogens, spread them between individuals which already may have suboptimal health [8].

Due to the observed vectorial importance of these facultative parasites, scientific interest in the potential role of more permanently symbiotic obligate ectoparasites has grown in the last decade. Included in this set of obligate parasites are blood-feeding bat ectoparasites. These comprise of bat flies, bat fleas, bat mites, biting midges, parasitic bugs, chiggers and ticks, all with different degrees of dispersal ability. Among these, ticks and chiggers often bite humans, with ticks transmitting both bacteria and viruses in their bites in the event that that they are infected. Although bat flies, bat fleas, bat mites, and the parasitic bugs specific to bats are not known to infest or bite human beings, their mobility (for winged Streblidae) and presence on the walls of caves frequented by human beings may represent a risk of biting. Thus understanding the role of ectoparasites in hosting and transmitting pathogens, as well as their potential links with habitat health has fundamental insights for understanding how to manage ecosystems effectively.

Human-infecting viruses in the families *Paramyxoviridae* (Nipah virus, Hendra virus, Menangle virus), *Filoviridae* (Marburg virus, Ebola virus), *Rhabdoviridae* (Australian bat lyssavirus), and *Coronaviridae* (SARS-CoV, MERS-CoV, SARS-CoV-2) are either of bat origin or probable bat origin, as inferred from serological or PCR based analysis of bat samples. Most of these pathogens are apparently non-pathogenic in their bat hosts. This has led to the suggestion that these infecting (or simply co-occurring) viruses have bats as their natural reservoir and thus exist at a state resembling equilibrium. Nevertheless, even viruses known to be non-pathogenic to bats can be transmitted to humans and other species, potentially resulting in observable disease. In this context, presence of infected bats particularly in shared habitats close to human habitations presents the risk of virus spillover to humans.

One preliminary step towards investigating the possible vectorial role for bat ectoparasites is the study of their viromes. Such a study would elucidate the potential role of these arthropods in maintenance and amplification of bat viruses within host communities. To elucidate the viruses present in bat ectoparasites, we conducted a metaviromic investigation of arthropods collected from bats in Yunnan Province, China.

2. Materials and methods

2.1. Sampling sites

Sampling in this study was carried out from July to November 2020

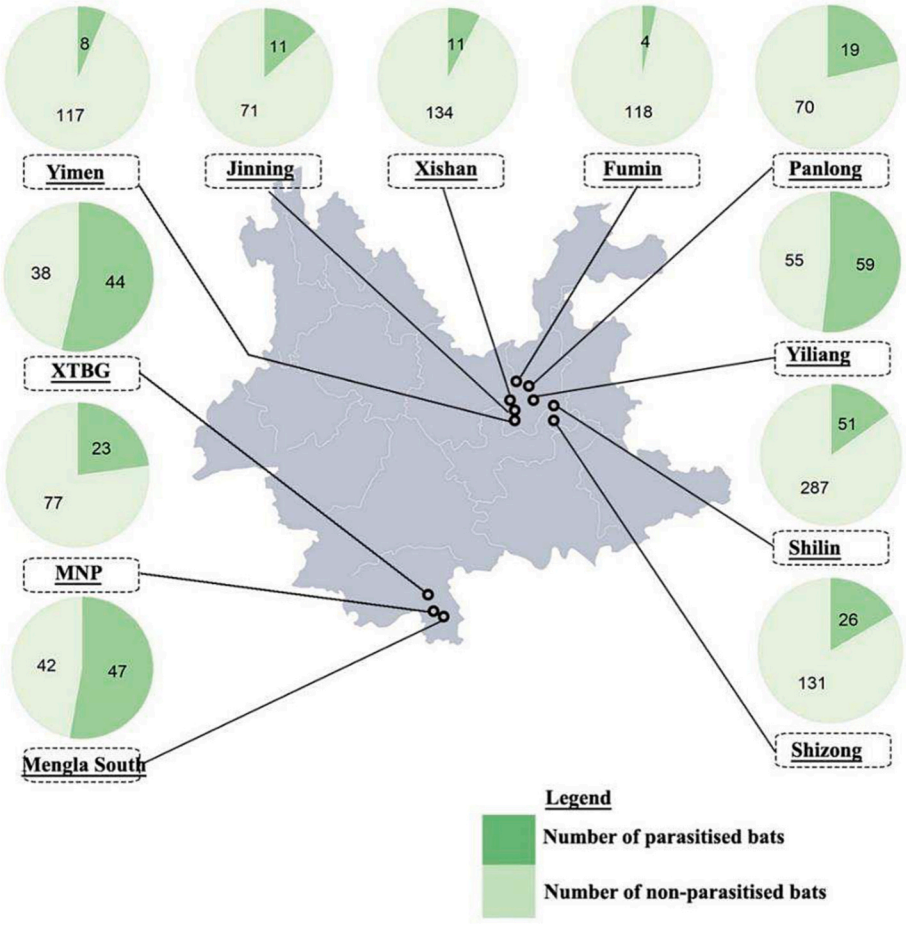

Fig. 1. Map of sampling sites in this study. Pie charts show the number of parasitized and non-parasitized bats per location.

at fifteen sampling locations in Yunnan Province, China. Sites were selected through both previous regional survey work by the group, and prospecting in areas and counties with known karsts (i.e., landforms underlain with limestone that has been eroded over time, producing cavern structures). The sampling sites included Xishan, Fumin, Yimen, Panlong, Yiliang, Shilin (3 sites), Shizong (2 sites), Jining, Mengyuan National Park (MNP), Xishuangbanna Tropical Botanical Garden (XTBG), and Mengla south (2 sites). See Fig. 1 and Tables 1-2. Each site was visited for sampling once, except for MNP, in which samples were taken twice at one month apart.

2.2. Ethical approval

The ethical approval for bat sample collection in Yunnan Province was provided by the Ethics Committee of Life Sciences, Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS) (approval number is SMKX-20200210-01). Sampling approval was provided from the provincial office in Jinghong, Xishuangbanna Nature Reserve office as well as from XTBG.

2.3. Bat and ectoparasite sampling

Bats were trapped using harp traps, mist nets and direct removal of bats from cave walls, where appropriate. Ectoparasites were detected by inspecting all external surfaces of the bat, and by moving fur in the opposite direction to the growth direction using a pair of forceps. In brief, all ectoparasites on each bat were picked using a disinfected pair of forceps before being preserved in M199 medium (supplemented with 50 μg/mL of both Kanamycin and Ampicillin). All samples were transferred to −80 °C within twelve hours and were further transported in dry ice from Yunnan Province to the Institut Pasteur of Shanghai- Chinese Academy of Sciences (IPS-CAS), where they were preserved at −80 °C pending further processing.

2.4. Bat and ectoparasite identification

Bats were identified morphometrically by qualified field biologists with prior experience in bat identification in South-East Asia. Tissue samples were also collected for later species validation [9,10]. All bats were kept in clean cotton bags and released after all measures were recorded.

To identify the families of ectoparasites in this study, a modified bioinformatic barcoding technique was conducted [11]. In the procedure, four genes were used for identification. They included the cytochrome *c* oxidase subunits I & II, cytochrome *b* and cytochrome *c*1. In the first step, all reads per ectoparasite pool were assembled into contigs. All contigs longer than 400 bases were then queried against the NCBI non-redundant protein database (NR) using BLASTx. The query was delimited within eight dipteran families including Streblidae, Nycteribiidae, Ischnopsyllidae, Cimicidae, Spinturnicidae, Trombiculidae, Ixodidae, and Argasidae (Taxonomy IDs: Table 3) whose members parasitize bats. The top hit for each contig was retained and the identity of its taxonomic family retrieved using Taxonkit [12]. To estimate the abundance of these ectoparasite families in each pool, all reads were mapped onto the productive contigs, and the read numbers normalized for ectoparasite numbers per pool.

The ectoparasite barcoding data was analyzed for all sampling sites (Supplementary Table 3) and for eight condensed sampling locations (Table 3) including Loc_a (Xishan), Loc_b (Shilin), Loc_c (Shizong), Loc_d (Yiliang), Loc_e (Panlong), Loc_f (Mengyuan national park), Loc_g (Mengla South), and Loc_h (XTBG). No threshold for read numbers was applied.

2.5. NGS library preparation

For sequencing library preparation, ectoparasites were pooled according to the sampling site and identity of the host. The specimens were retrieved from −80 °C, thawed at 4 °C and each transferred into a fresh 1.5 mL Eppendorf tube along with the viral transport medium (VTM). A single sterile 4.5 mm steel bead in each tube was then used to grind the specimens in a tissue homogenizer for three minutes at 60 Hz. A 1 mL volume of homogenate consisting of equal portions from tubes of the same pool was then constituted, by transferring those portions into a fresh cryogenic tube. The pooled homogenates were then centrifuged at 13,400 ×*g* for 3 min at 4 °C and the supernatants transferred into a fresh Eppendorf tube. These tubes were centrifuged at 6000 ×*g* for 3 min, with this step being repeated in a new tube in case of filter blockage. From the filtrate, viral RNA purification was performed using the GeneJET Viral DNA and RNA purification kit (Thermo Scientific Cat No. K0821), following manufacturer instructions. Non-selective transcriptome amplification was then performed using the Sigma-Aldrich Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich Cat No. WTA2) following manufacturer instructions, and the PCR products purified using the Monarch PCR and DNA Cleanup kit (Cat No. NEB T1030).

Eight Microliters of DNA were then used as template for the NEBNext® Ultra™ II Library Prep Kit for Illumina (Cat No. E7645S). All manufacturer instructions were followed except for the use of AMPure XP Beads (in place of the recommended NEBNext Sample Purification beads). The AMPure XP beads were utilized for cleanup of adaptor-ligated DNA fragments and for DNA cleanup following the final amplification step in the NEBNext Ultra II Library Prep protocol. To combine all samples into sequencing pools as potentiated by the numerous library indices available, the DNA concentration for all samples was determined using the Qubit™ 1× ds High Sensitivity Assay kit and the Qubit 3.0 fluorometer. The superpools generated during library preparation were then sequenced on an Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) to yield paired-end reads of 150 bp in length.

2.6. Analysis of sequencing reads and taxonomic classification

The sequencing reads from all pools were analyzed via two pipelines: namely the VHF-NGS pipeline and the PIMGAVir NGS pipeline

Table 1
Sampling details. Parasitized and non-parasitized bats sorted by sampling location (male/female/unidentified sex shown in parentheses).

Sampling Location	Parasitized bats (no.) (M, F, NA)	Non-parasitized bats (no.) (M, F, NA)	Total bats (no.) (M, F, NA)	Parasitized bats (%)	Bat species (no.)
Fumin	4 (2,2,0)	118 (27,23,68)	122 (29,25,68)	3.28	14
Jining	11 (3,8,0)	71 (29,41,1)	82 (32,49,1)	13.41	5
Mengla_South	47 (28,19,0)	42 (17,25,0)	89 (45,44,0)	52.81	6
Mengyuan	23 (20,3,0)	77 (34,43,0)	100 (54,46,0)	23.00	4
Panlong	19 (7,12,0)	70 (46,24,0)	89 (53,36,0)	21.35	7
Shilin	51 (28,23,0)	287 (122,166,1)	338 (148,189,1)	15.09	12
Shizong	26 (9,17,0)	131 (62,68,1)	157 (71,85,1)	16.56	10
Xishan	11 (5,6,0)	134 (54,78,2)	145 (59,84,2)	7.59	9
XTBG	44 (13,9,22)	38 (11,18,9)	82 (24,27,31)	53.66	13
Yiliang	59 (32,27,0)	55 (26,29,0)	114 (58,56,0)	51.75	6
Yimen	8 (5,3,0)	117 (47,64,6)	125 (52,67,6)	6.40	9

Table 2
Sampling details. Parasitized and non-parasitized bats sorted by bat families.

Bat Species	Parasitized bats (no.)	Non-parasitized bats (no.)	Total bats (no.)	Parasitized bats (%)	Range of ectoparasite (no.) per individual
Aselliscus stoliczkanus	8	56	64	12.5	1–5
Hipposideros armiger	1	40	41	2.44	1
Hipposideros larvatus	1	35	36	2.78	5
Hipposideros pomona	5	54	59	8.47	1–3
Cynopterus brachyotis	2	0	2	100	2–6
Eonycteris spelaea	46	45	91	50.55	1–5
Rousettus amplexicaudatus	1	0	1	100	2
Ia io	0	1	1	0	0
Myotis chinensis	0	1	1	0	0
Myotis fimbriatus	21	113	134	15.67	1–5
Myotis laniger	16	198	214	7.48	1–4
Myotis pilosus	17	13	30	56.67	1–5
Myotis siligorensis	3	2	5	60	1–2
Myotis sp.	1	5	6	16.67	1
Pipistrellus spp.	0	2	2	0	0
Pipistrellus spp2	0	1	1	0	0
Rhinolophus affinis	49	143	192	25.52	1–14
Rhinolophus malayanus	11	18	29	37.93	1–3
Rhinolophus marshalli	0	2	2	0	0
Rhinolophus pusillus	3	25	28	10.71	1
Rhinolophus rex	2	12	14	14.29	1
Rhinolophus siamensis	3	39	42	7.14	1
Rhinolophus sinicus	71	248	319	22.26	1–4
Rhinolophus stheno	0	1	1	0	0
Rhinolophus thomasi	1	0	1	100	1
Megaderma lyra	2	0	2	100	10–17
Miniopterus schreibersii	39	86	125	31.2	1–8

Table 3
Number of normalized read numbers that mapped onto the host cytochrome gene markers (normalized for ectoparasite number per location). Corresponds to Supplementary Fig. 4.

	NCBI Taxonomy ID	Loc_a (Xishan)	Loc_b (Shilin)	Loc_c (Shizong)	Loc_d (Yiliang)	Loc_e (Panlong)	Loc_f (Menyuan)	Loc_g (Mengla)	Loc_h (XTBG)
No. of Ectoparasites		12	68	11	67	1	3	18	48
Nycteribiidae (bat flies)	81707	6802.214	126,186.9	438,011.7	11,309.29	11,309.29	0	0	14,004.76
Spinturnicidae (bat mites)	99230	0	0	4079.143	21,322.73	21,322.73	301,838	0	32,652.41
Streblidae (bat flies)	81697	2	12,019.68	5377	2045.428	2045.428	7	5249.333	26,001
Ixodidae (hard ticks)	6939	402.25	1987.559	14,249.86	1598.504	1598.504	0	0	1209.643
Trombiculidae (Chiggers)	92251	3745.5	456.2646	18,488.57	2454.217	2454.217	198	0	688.6931
Cimicidae (bed bugs)	30078	0.428571	408.9231	6715	33.2697	33.2697	0	34.33333	55.58333
Ischnopsyllidae (bat fleas)	140,720	19.5	289.2378	0	272.9	272.9	0	14.66667	3.125
Argasidae (soft ticks)	6936	2	296.8974	85	0.882576	0.882576	0	0	0

(Supplementary Fig. 2). The VHF-NGS pipeline is described in Supplementary File 1, and PIMGAVir pipeline has been described elsewhere [13]. The viral hits simultaneously identified by both pipelines were carried into subsequent validation steps.

3. Results

3.1. Sampled bat species

A total of 1443 bats from five bat families were trapped and sampled in this study. These included 27 species within Rhinolophidae (628 individuals), Vespertilionidae (519), Hipposideridae (200), Pteropodidae (94), and Megadermatidae (2) (Table 2).

In total 303 (21%) out of 1443 bats sampled were parasitized, with the remaining 1140 (79%) found without ectoparasites. As shown in Supplementary Table 5, male bats (152/625) were significantly more parasitized that female bats (129/708) [$\chi 2$ $p < 0.05$]. The most parasitized bats by number were Rhinolophidae (140 individuals) and Vespertilionidae (97). It was found that Hipposideridae (15/200–7.5%) were the least parasitized bat family of all five (Fisher's Exact test; $p < 0.05$. See Supplementary Table 4). Although Megadermatidae (2/2) were significantly more parasitized than Hipposideridae and Vespertilionidae, the number of Megadermatidae bats sampled were notably low.

The most frequently parasitized bat species were *Rhinolophus sinicus* (71/319 individuals), *Rhinolophus affinis* (49/192), *Eonycteris spelaea* (46/91), *Miniopterus schreibersii* (39/125), and *Myotis fimbriatus* (21/134). By proportion, other than in the low abundance species in which all bats were parasitized, *Myotis pilosus* (17/30–56.6%), *Eonycteris spelaea* (46/91–50.5%) and *Rhinolophus malayanus* (11/29–37.9%) were highly parasitized. Out of 27 bat species sampled, six species (*Rhinolophus marshalli*, *Rhinolophus stheno, Ia io, Myotis chinensis*, and both unidentified *Pipistrellus* spp.) were found without ectoparasites.

The highest total number of ectoparasites were found in *M. schreibersii* (76), while the highest number of ectoparasites on single hosts were found on *Megaderma lyra* (17) and *Rhinolophus affinis* (14). Ectoparasite numbers per bat ranged between 1 and 17 with an overall ectoparasite intensity of 1.76. This is likely to relate to interspecific interactions, in addition to grooming, and may also link to individual health.

3.2. Bat ectoparasite species

The data for 228 ectoparasites collected from 121 sampled bats were analyzed in this study. The distribution of these ectoparasites across sampling sites and host species are shown in Table 3, Supplementary Table 1 and Supplementary Table 2.

As seen in Table 3 and Supplementary Table 3 & 5, the sequencing reads mapped onto all eight families analyzed in this study including Streblidae, Nycteribiidae, Ischnopsyllidae, Cimicidae, Spinturnicidae, Trombiculidae, Ixodidae, and Argasidae. There was a predominantly large number of reads mapping onto the Nycteribiidae cytochrome genes. In 20 out of 31 pools, most reads were of Nycteribiidae origin. The second most abundant ectoparasite family was Spinturnicidae, a mite family parasitic on bats. These mites represented the most abundant ectoparasite family in 3 of the 31 pools. Streblidae bat flies were the third most abundant family, accounting for approximately 11% of the reads mapped onto Nycteribiidae bat flies. Of the two tick families evaluated, Ixodidae (hard-bodied ticks) were more abundant than Argasidae (soft-bodied ticks), with Argasidae representing the least abundant of the eight ectoparasite families. The Trombiculidae (chiggers) were the most abundant ectoparasite in pool 23 (*M. schreibersii*, Loc_g) and showed a notably high number of reads in pools 3 (*R. sinicus*, Loc_a), 12 (*M. pilosus*, Loc_c), and 17 (*M. schreibersii*, Loc_d). For the Ischnopsyllidae (bat fleas) family, reads were identified only in 6 of the 31 pools and Cimicidae (parasitic bat bug) reads were identified only in 8 of the 31 pools.

3.3. Viral abundance in pooled ectoparasites

The data for 121 out of 303 parasitized bats were included in this study (Supplementary Tables 1 & 2). For each of the contigs that were found to have >70% sequence homology to viral proteins in the NR database (Fig. 2 shows proportions of virus abundance estimated from mapped reads), all reads in the given pool were mapped onto said contigs to produce an estimate of abundance. Most of the contigs yielded hits of bacteriophage origin upon a sequence homology search on the NCBI NR protein database and these were not included in the abundance estimation (Supplementary Fig. 3b).

Viral hits of *Reoviridae* origin were found across all eight locations (Fig. 2a). The second most ubiquitous viral families included

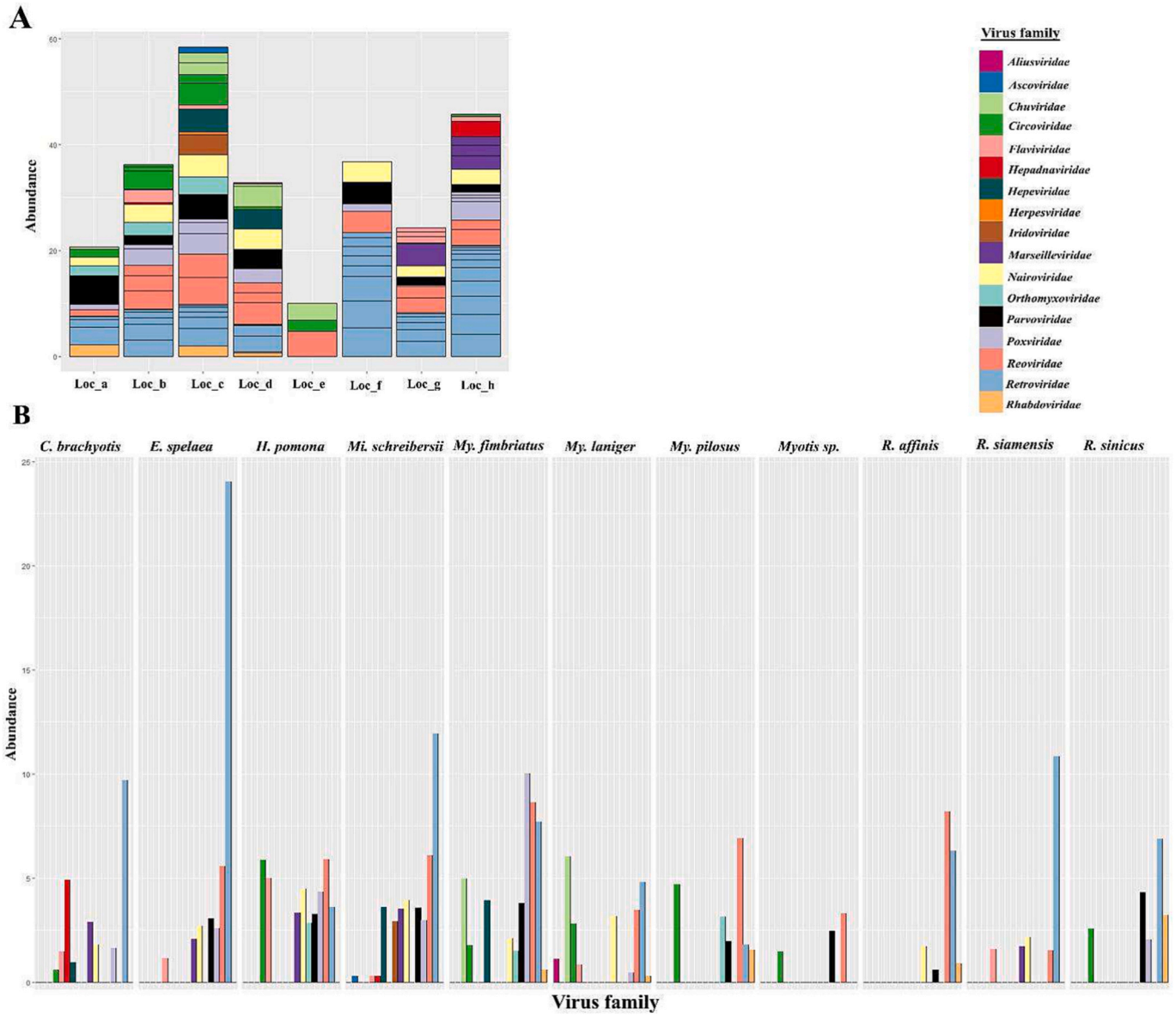

Fig. 2. Viral abundance by location (A) and sampled bat host species (B).

Retroviridae, Parvoviridae, Poxviridae, and *Nairoviridae*, all of which were present in seven of the eight locations. Other viral families represented in the sampling locations were *Circoviridae, Chuviridae, Flaviviridae, Rhabdoviridae, Orthomyxoviridae, Marseilleviridae, Iridoviridae, Hepeviridae, Herpesviridae, Hepadnaviridae, Aliusviridae*, and *Ascoviridae*. Shizong county (Loc_c) was found to have the highest variety of viral families, with 14 of the 17 families evaluated being found in this location. Other locations with high numbers of virus families included Yiliang (loc_d; 10/17), Xishan (loc_a; 9/17), Shilin (loc_b; 9/17), and XTBG (loc_h; 9/17). Other sampling sites contained fewer viral families: MXZ (loc_g; 7/17), Mengyuan (loc_f; 5/17), and Panlong (loc_e; 3/17).

3.4. Tentative virus families identified by both analysis pipelines

Cumulatively, 2554 and 2426 assembled contigs were independently predicted to contain virus or virus-like genes by PIMGAVir and VHF-NGS pipelines respectively. The boxplot in Supplementary Fig. 3a shows the range of predicted viral contigs per pool from either pipeline. These predictions were based on the viruses (Taxid: 10239) taxonomic node of the NCBI NR protein database. Of these, 438 contigs were simultaneously predicted by both pipelines as having virus or virus-like genes. Only two of the predicted viral genes were homologous to mammalian and arthropod infecting viruses with the rest of the predicted viruses being viruses of bacteria, algae, and plants (bacteriophages, >93%). Supplementary Fig. 3b shows the distribution of these bacteriophage contigs by length and mean depth of mapped reads.

The two contigs with significant homology to mammalian and arthropod viruses were derived from pools 24 and 27, which consisted of ectoparasite homogenates for bat flies found infesting *Eonycteris spelaea* in Mengla south and XTBG, respectively. As shown in Supplementary Table 1, most reads mapped onto Nycteribiidae Cytochrome genes. Pool 27 had comparable read numbers mapping onto Ixodidae and Streblidae genes, suggesting that members of these bat-fly families were the source of the identified viral genes. In pool 24, one contig (length = 416 bp) was found to be homologous to the coding region for the Parvovirus VP1/VP2 gene. This sequence showed 78.8% nucleotide sequence identity to the VP1/VP2 gene of *Rhinolophus affinis bocaparvovirus* (GenBank accession: MG986723.1). Phylogenetic analysis (Fig. 3) consisting of VP1/VP2 parvovirus genes from similar sequences with >80% nucleotide identity (also >80% query cover) to our contig showed this gene, provisionally named Nycteribiidae bat-fly bocaparvovirus VP1/VP2 to be most closely related to the VP1/VP2 gene of *Rhinolophus affinis bocaparvovirus* identified from *Rhinolophus affinis* in Puer, Yunnan Province, China in 2016 (GenBank accession: MG986723.1; Taxid: 2053082). All closely related gene sequences derive from the *Bocaparvovirus* genus of the *Parvovirinae* sub-family, whose members have been identified from mammalian hosts.

In pool 27 (from probable Streblidae and Ixodidae parasitizing *Eonycteris spelaea* in XTBG), a 171 bp region of a 915 bp long contig showed 98.2% amino acid identity to a ubiquitin-like protein of Flamingopox virus (NCBI accession: YP_009447989.1; Taxid 2,059,380). Phylogenetic analysis with similar sequences (>80% amino acid identity, >18% query cover) (SF-1) showed our candidate ubiquitin-like protein, provisionally named Bat-fly candidate poxvirus ubiquitin-like protein to form a monophyletic group with Canarypox virus (NCBI accession: NC_005309.1; Taxid: 44088) and Shearwaterpox virus strain SWPV-2 (NCBI accession: KX85721.1; Taxid: 1974596). (Supplementary Fig. 1).

4. Discussion

4.1. Viruses on bat ectoparasites parasitizing different bat hosts

Whilst ectoparasite prevalence will vary with host, location, season,

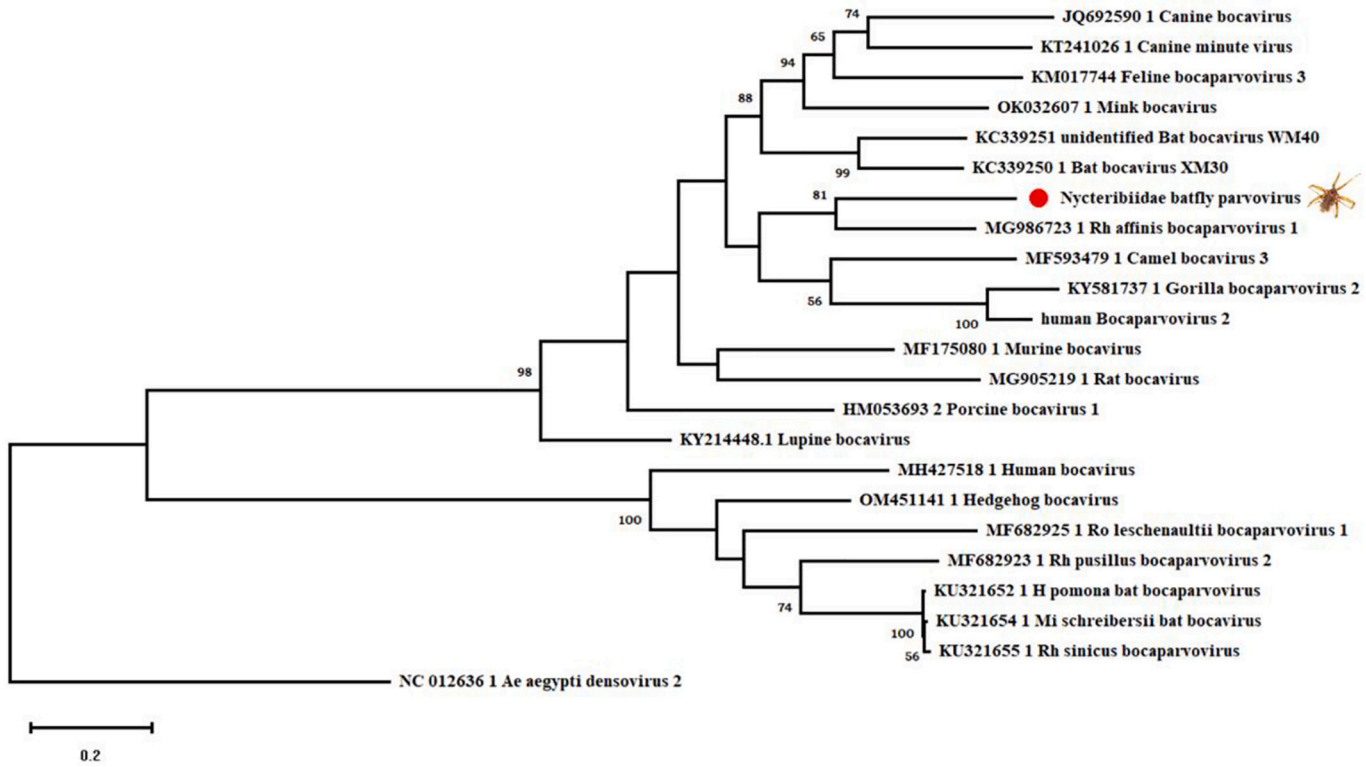

Fig. 3. Maximum likelihood phylogenetic tree of Parvoviruses based on the VP1/VP2 gene. Virus names are preceded by their NCBI GenBank accession number. The candidate parvovirus identified in this study is indicated by a red dot, with a bat fly. Numbers beside the nodes represent statistical confidence in clades based on 1000 bootstrap replicates; only bootstrap values ≥50% are shown. The scale bar represents nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

health and reproductive status, sex and various other factors, most studies correspond in terms of what ectoparasites are common on many bats. Contrary to previous findings regarding the comparable and generally high abundance of Streblidae and Nycteribiidae bat flies on bats [14], our data indicate a higher abundance of Spinturnicidae than Streblidae bat flies in our captured animals. Spinturnicidae are mites known to infest the wing membrane of bats and could move between nearby individual bats. The high incidence of these mites on the wings of *Myotis*, and *Eonycteris* but not *Hipposideros* or *Rhinolophus* may relate to roosting patterns (close contact which provides an easy means to spread viruses between hosts), as outside hibernation *Rhinolophus* and *Hipposideros* generally roost at greater inter-individual distances (*pers obs*). It may be that these mites are common on these four bat families, but their small size and lower mobility compared to bat flies prevents easy detection, whereas here we explicitly looked for these mites. The high abundance of reads mapping onto Nycteribiidae genes is also consistent with the higher frequency of Nycteribiidae species in the eastern rather than the western hemisphere as compared to Streblidae species [15].

Although ectoparasites from *Mi. schreibersii, My. fimbriatus, My. laniger* and *C. brachyotis* have an apparent higher richness in predicted viral families (though the sample size is small), this does not correlate with ectoparasite number, as these four host species showed widely disparate ectoparasite numbers. Our data show that although ectoparasite numbers and ectoparasite family richness (based on raw rather than relative numbers) are comparable in *C. brachyotis* and the unidentified *Myotis* sp., their richness in predicted virus families appears slightly different (Fig. 2B, Supplementary Fig. 5). This suggests a higher propensity for the hosting of viruses by Nycteribiidae as opposed to Spinturnicidae ectoparasites, and the variable mobility of these parasites as well as their host choice has implications for the spread of viruses. Furthermore, ticks (e.g., Ixodidae) are generalist parasites, and can move between bat and non-bat hosts, and could act to move pathogens between mammalian species.

Tentative viral contigs homologous (>80% amino acid identity) to *Retroviridae* proteins were found in all but one of all eight sampling sites. *Retroviridae* hits were also present and abundant in all but one of all 11 host species from which ectoparasites were collected. The fact that none of these had extensive portions of their genomes recovered suggests that these are endogenous retroviral elements in the ectoparasite genomes. The observation, however, that retroviruses constitute only trace proportions of integrated viruses in insect genomes [16] suggests that these may also be of bat-origin. In our analysis, *Retroviridae* reads are present in ectoparasite pools from all bat species except the unidentified Myotis sp., which happens to have the highest relative abundance of Spinturnicidae (Supplementary Table 1). If the predicted *Retroviridae* in this study are of bat origin, this observation from the *Myotis* sp., suggests that *Retroviridae* from bats are more effectively acquired from the bats by non- Spinturnicidae ectoparasites. Other predicted viruses of probable bat origin are the Parvoviridae and Poxviridae present in ectoparasites found on *My. fimbriatus.* This is supported by previous data in which we showed the presence of Parvoviridae and Poxviridae sequences in *My. fimbriatus* rectal swabs [17].

Some *Reoviridae*, *Nairoviridae* and *Rhabdoviridae* members are arthropod-specific viruses. To the best of our knowledge, no bat-fly specific viruses in these three families have been identified. Here, representing tentative reoviral sequences were identified in all sampling sites and nine of the eleven bat species. Reoviruses have recently been reported from possible Streblidae bat flies parasitizing *Pternotus parnellii* in Mexico [18]. An orthoreovirus was also identified in *Eucampsipoda africana* parasitizing *Rousettus aegyptiacus* bats in South Africa, where the identified virus formed syncytia and caused cytopathic damage after three blind passages in VeroE6 cells [19]. *Reoviridae* members have been shown to replicate in arthropods as exemplified by the transmission of Colorado tick fever virus by *Dermacentor andersoni* and Bluetongue virus by biting midges (*Cullicoides obsoletus*) [20–22].

Nairoviridae homologous contigs, indicating the possible presence of *Nairoviridae*, were retrieved from seven of eight sampling sites and ectoparasites parasitizing eight of eleven sampled bat species. Although no *Nairoviridae* have previously been reported from bat associated ectoparasites, other members of the *Bunyavirales* order have been detected in bat flies. These include the identification of Kaeng Khoi virus following the culture of bedbug- (*Stricticimex parvus*) derived homogenates in Thailand [23] and *Eucampsipoda sundaica*-derived homogenates in BHK [24] and VeroE6 [25] cells in China. These ectoparasites were found parasitizing *Tadarida plicata* and *Rousettus leschenaultii* in those respective countries. Additionally, soft tick bunyavirus and a novel orthobunyavirus were detected in cell culture from *Argas vespertilionis* (tick) [26] and *Eucampsipoda sundaica* (bat-fly, family; *Nycteribiidae*) [27] homogenates, respectively. More recently, a novel *Peribunyaviridae* was identified by NGS from bat-flies parasitizing *Hipposideros ruber* in Uganda [18]. All of these *Bunyavirales* members were detected from ectoparasites parasitizing apparently healthy bats, and their virulence in host species were not determined.

Our study showed the possible presence of rhabdoviruses in bat-associated ectoparasites collected in three of eight sampling sites, as well as five of the eleven sampled bat species. These include *Myotis fimbriatus*, *M. laniger, M. pilosus*, *Rhinolophus affinis*, and *R. sinicus* trapped at Loc_a (Xishan), Loc_c (Shizong), and Loc_d (Yiliang). Our analysis showed that Nycteribiidae (28.9–89.9%), Trombiculidae (3.7–34.1%), and Spinturnicidae (0.83–54.6%) arthropod host markers were highly prevalent in most of the locations. This result is consistent with the identification of rhabdoviral sequences in Nycteribiidae bat flies parasitizing *Myotis daubentonii* in Spain [28] and both *Lissonycteris angolensis ruwenzorii* and an unidentified *Myonycteris spp* in Uganda [29,30]. In the former study, RT-PCR of the rhabdovirus L gene indicated the presence of rhabdovirus sequences in all nine ectoparasite pools. As all Nycteribiidae in that study were found to be positive for rhabdoviral sequences, it was hypothesized that the bat flies were either infected by rhabdoviruses or the high positivity rate was the result of endogenous viral elements (EVE) detection. Nevertheless, our study shows that in addition to Nycteribiidae bat flies, bat ectoparasites of the families Trombiculidae (chiggers), and Spinturnicidae (bat mites) may act as hosts for rhabdoviruses. Whether these are of bat-origin or represent insect-specific rhabdoviruses remains to be studied.

Contigs homologous to *Flaviviridae* hits were found in *Cynopterus brachyotis, Eonycteris spelaea, Hipposideros pomona, Myotis laniger*, and *Rhinolophus siamensis*. Among *Flaviviridae*, only Dengue virus (DENV) has been found in infected bat-flies, as confirmed by RT-PCR. These were however collected from *Desmodus rotundus* (family; Phylostomidae) where *Strebla wiedemanni* and *Trichobius parasiticus* bat flies (both of Streblidae family) were hypothesized as being reservoirs for the sylvatic transmission of DENV. This speculation requires further study. Tentative flaviviral sequences were detected in ectoparasite pools from Shilin (loc_b), Shizong (loc_c), MXZ (loc_g), and XTBG (loc_h), where Nycteribiidae, Streblidae, and Spinturnicidae host markers were most abundant (Fig. 2a). Besides confirming the presence of *Flaviviridae* in Streblidae bat flies, our findings indicate the presence of *Flaviviridae* sequences in two other bat ectoparasite families: Nycteribiidae and Spinturnicidae.

4.2. Putative virus genes identified from bat ectoparasites in this study

The Nycteribiidae bat-fly bocaparvovirus described in this study appears to cluster together with mammalian viruses, with its closest match having been identified from *Rhinolophus* bats. Nycteribiidae can be particularly large parasites, and as they are consumed during grooming (including those in the study-sites; Data not shown), this provides another potential means to transmit viruses between bats. This Nycteribiidae bat-fly bocaparvovirus and its closest match together form a clade that is most closely related to viruses identified in primates (Gorilla and human) [34], and all closely related virus sequences (>80% nucleotide sequence identity) are derived from mammal associated

viruses. This evidence points to the acquisition of the Nycteribiidae bat-fly bocaparvovirus from bats by these ectoparasites. However, because no similar contigs were found in ectoparasites of bats in the same roost, no vector or mechanical transmission may be ascribed to the bat-flies in this pool. There is also no evidence of bocaparvovirus replication in bat flies as all *Parvoviridae* identified in arthropods are in the *Densovirinae* sub-family. Multiple members of the *Densovirinae* sub-family are insect-specific viruses of mosquitoes, in which they have been shown to replicate productively [31–33]. None of the parasitized bats in our study exhibited signs of disease, which is consistent with the identification of parvoviruses from both healthy and unhealthy non-human primates [35]. Nevertheless, insect-specific viruses with the potential to infect mammals, and whose genetic sequences are currently incompletely characterized, may contribute genetically to the emergence of novel mammalian viruses. Understanding the roles of ectoparasites in transmission of viruses is crucial if we are to understand their dynamics in colonies, and the intersection with individual and habitat health.

5. Conclusions

We explored the potential of parasites as vectors of bat-borne viruses. We showed that both specialist and generalist parasites carry a variety of viruses, and may provide a hitherto under-appreciated mechanism of transmission between bats and from bats, to human beings, who come into contact with bats or enter bat dwellings. The distribution of parasites shows linkages to bat behaviour, and through the movement of parasites as well as consumption via allogrooming could provide a direct means of transmission of certain viruses. Generalist ectoparasites may also provide a means of transmitting pathogens between bats and other mammals sharing their habitats. Differences in parasite load were noted between males and females (though differences in parasite viral load by sex were not found). Males were observed to host many more parasites in a number of species, possibly reflecting different levels of interactions and requiring more work to understand how these differences in parasite load reflect behaviour and ecophysiology. Further work is needed to establish how dynamics change across space and time, as well as with habitat complexity, as parasite load and transmission may vary under these conditions. Whilst we observed differences in parasite and viral load in each site, further work and more samples would be needed to understand how these trends vary based on the bat community, time of year and the structure of the landscape.

Author contributions

Conceptualization: GW, ACH and NB. Funding acquisition and supervision: GW and NB. Methodology and data curation: AT, YK, RL, XC, YC, VO, EM and ACH. Data analysis: AT, JL, NB, ACH, and GW. Writing (1st draft): AT. Writing (revision): GW, ACH, JL and NB.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The raw reads were deposited at NCBI (BioProject Accession number PRJNA940154).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2023.100641.

References

[1] Y.M. Bar-On, R. Phillips, R. Milo, The biomass distribution on Earth, Proc. Natl. Acad. Sci. 115 (25) (Jun. 2018) 6506–6511, https://doi.org/10.1073/pnas.1711842115.
[2] A.J. Hamilton, et al., Estimating global arthropod species richness: refining probabilistic models using probability bounds analysis, Oecologia 171 (2) (2013) 357–365, https://doi.org/10.1007/s00442-012-2434-5.
[3] L. Harrison, The Mallophaga as a possible clue to bird phylogeny, Aust. Zool. 1 (1) (1914) 7–11.
[4] T.M. Goater, C.P. Goater, G.W. Esch, Parasitism: The Diversity and Ecology of Animal Parasites, Cambridge University Press, 2014.
[5] A. Abdel-Wahab, et al., Dynamics of gametocytes among Plasmodium falciparum clones in natural infections in an area of highly seasonal transmission, J. Infect. Dis. 185 (12) (Jun. 2002) 1838–1842, https://doi.org/10.1086/340638.
[6] G.M. Jeffery, D.E. Eyles, Infectivity to mosquitoes of plasmodium falciparum as related to gametocyte density and duration of infection, Am. J. Trop. Med. Hyg. 4 (5) (Sep. 1955) 781–789, https://doi.org/10.4269/ajtmh.1955.4.781.
[7] M.J. Valentine, C.C. Murdock, P.J. Kelly, Sylvatic cycles of arboviruses in non-human primates, Parasit. Vectors 12 (1) (2019) 1–18.
[8] T. Postawa, Z. Nagy, Variation of parasitism patterns in bats during hibernation: the effect of host species, resources, health status, and hibernation period, Parasitol. Res. 115 (10) (Oct. 2016) 3767–3778, https://doi.org/10.1007/s00436-016-5138-7.
[9] A. Chornelia, J. Lu, A. Hughes, How to accurately delineate morphologically conserved taxa and diagnose their phenotypic disparities: species delimitation in cryptic Rhinolophidae (Chiroptera), Front. Ecol. Evol. 10 (Mar. 2022) 854509, https://doi.org/10.3389/fevo.2022.854509.
[10] A. Chornelia, A.C. Hughes, Phenotypic traits evolution and morphological traits associated with echolocation calls in cryptic horseshoe bats (Rhinolophidae), J. Syst. Evol. n/a (n/a) (Oct. 2022), https://doi.org/10.1111/jse.12924.
[11] Z. Xu, et al., Virome of bat-infesting arthropods: highly divergent viruses in different vectors, J. Virol. 96 (4) (Feb. 2022) e0146421, https://doi.org/10.1128/JVI.01464-21.
[12] W. Shen, H. Ren, TaxonKit: a practical and efficient NCBI taxonomy toolkit, J. Genet. Genomics 48 (9) (Sep. 2021) 844–850, https://doi.org/10.1016/j.jgg.2021.03.006.
[13] E. Mastriani, K.M. Bienes, G. Wong, N. Berthet, PIMGAVir and Vir-MinION: two viral metagenomic pipelines for complete baseline analysis of 2nd and 3rd generation data, Viruses 14 (6) (Jun. 2022), https://doi.org/10.3390/v14061260.
[14] C.W. Dick, K. Dittmar, in: S. Klimpel, H. Mehlhorn (Eds.), Parasitic Bat Flies (Diptera: Streblidae and Nycteribiidae): Host Specificity and Potential as Vectors BT - Bats (Chiroptera) as Vectors of Diseases and Parasites: Facts and Myths, Springer Berlin Heidelberg, Berlin, Heidelberg, 2014, pp. 131–155.
[15] C.W. Dick, R. Pospischil, in: H. Mehlhorn (Ed.), Nycteribiidae (Bat Flies) BT - Encyclopedia of Parasitology, Springer Berlin Heidelberg, Berlin, Heidelberg, 2014, pp. 1–4.
[16] C.D. Blair, K.E. Olson, M. Bonizzoni, The widespread occurrence and potential biological roles of endogenous viral elements in insect genomes, Curr. Issues Mol. Biol. 34 (2020) 13–30, https://doi.org/10.21775/cimb.034.013.
[17] A. Armero, et al., Myotis fimbriatus virome, a window to virus diversity and evolution in the genus myotis, Viruses 14 (9) (Aug. 2022), https://doi.org/10.3390/v14091899.
[18] M.M. Ramírez-Martínez, A.J. Bennett, C.D. Dunn, T.M. Yuill, T.L. Goldberg, Bat flies of the family Streblidae (Diptera: Hippoboscoidea) host relatives of medically and agriculturally important 'bat-associated' viruses, Viruses 13 (5) (2021), https://doi.org/10.3390/v13050860.
[19] P. Jansen van Vuren, et al., Isolation of a novel fusogenic orthoreovirus from Eucampsipoda africana bat flies in South Africa, Viruses 8 (3) (Feb. 2016) 65, https://doi.org/10.3390/v8030065.
[20] L.E. Rozeboom, W. Burgdorfer, Development of Colorado tick fever virus in the Rocky Mountain wood tick, Dermacentor andersoni, Am. J. Epidemiol. 69 (2) (1959) 138–145.
[21] C. De Liberato, et al., Identification of Culicoides obsoletus (Diptera: Ceratopogonidae) as a vector of bluetongue virus in Central Italy, Vet. Rec. 156 (10) (Mar. 2005) 301–304, https://doi.org/10.1136/vr.156.10.301.

[22] R.M. Du Toit, The Transmission of Blue-Tongue and Horse-Sickness by Culicoides, 1944.
[23] J.E. Williams, S. Imlarp, F.H. Top Jr., D.C. Cavanaugh, P.K. Russell, Kaeng Khoi virus from naturally infected bedbugs (cimicidae) and immature free-tailed bats, Bull. World Health Organ. 53 (4) (1976) 365–369 [Online]. Available, https://pubmed.ncbi.nlm.nih.gov/1086729.
[24] Z. Xu, et al., Isolation and identification of a highly divergent Kaeng Khoi virus from bat flies (Eucampsipoda sundaica) in China, Vector-Borne Zoonotic Dis. 19 (1) (Dec. 2018) 73–80, https://doi.org/10.1089/vbz.2018.2350.
[25] Y. Feng, et al., Isolation of Kaeng Khoi virus (KKV) from Eucampsipoda sundaica bat flies in China, Virus Res. 238 (Jun. 2017) 94–100, https://doi.org/10.1016/j.virusres.2017.06.007.
[26] M. Oba, et al., A novel Bunyavirus from the soft tick, Argas vespertilionis, in Japan, J. Vet. Med. Sci. 78 (3) (Mar. 2016) 443–445, https://doi.org/10.1292/jvms.15-0536.
[27] P. Jansen van Vuren, et al., Isolation of a novel orthobunyavirus from bat flies (Eucampsipoda africana), J. Gen. Virol. 98 (5) (May 2017) 935–945, https://doi.org/10.1099/jgv.0.000753.
[28] C. Aznar-Lopez, et al., Detection of rhabdovirus viral RNA in oropharyngeal swabs and ectoparasites of Spanish bats, J. Gen. Virol. 94 (Pt 1) (Jan. 2013) 69–75, https://doi.org/10.1099/vir.0.046490-0.
[29] A.J. Bennett, A.C. Paskey, J.H. Kuhn, K.A. Bishop-Lilly, T.L. Goldberg, Diversity, transmission, and Cophylogeny of Ledanteviruses (Rhabdoviridae: Ledantevirus) and Nycteribiid bat flies parasitizing Angolan soft-furred fruit bats in Bundibugyo District, Uganda, Microorganisms 8 (5) (May 2020), https://doi.org/10.3390/microorganisms8050750.
[30] T.L. Goldberg, A.J. Bennett, R. Kityo, J.H. Kuhn, C.A. Chapman, Kanyawara virus: a novel Rhabdovirus infecting newly discovered Nycteribiid bat flies infesting previously unknown Pteropodid bats in Uganda, Sci. Rep. 7 (1) (Jul. 2017) 5287, https://doi.org/10.1038/s41598-017-05236-w.
[31] K. Sangdee, S.-N. Pattanakitsakul, New genetic variation of Aedes albopictus densovirus isolated from mosquito C6/36 cell line, Southeast Asian J. Trop. Med. Public Health 43 (5) (Sep. 2012) 1122–1133.
[32] K. Sangdee, S.-N. Pattanakitsakul, Comparison of mosquito densoviruses: two clades of viruses isolated from indigenous mosquitoes, Southeast Asian J. Trop. Med. Public Health 44 (4) (Jul. 2013) 586–593.
[33] Y.-G. Zhai, et al., Isolation and characterization of the full coding sequence of a novel densovirus from the mosquito Culex pipiens pallens, J. Gen. Virol. 89 (Pt 1) (Jan. 2008) 195–199, https://doi.org/10.1099/vir.0.83221-0.
[34] A. Kapoor, et al., Identification and characterization of a new bocavirus species in gorillas, PLoS One 5 (7) (Jul. 2010) e11948, https://doi.org/10.1371/journal.pone.0011948.
[35] C.L. Byington, et al., Community surveillance of respiratory viruses among families in the Utah better identification of germs-longitudinal viral epidemiology (BIG-LoVE) study, Clin. Infect. Dis. 61 (8) (Oct. 2015) 1217–1224, https://doi.org/10.1093/cid/civ486.