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RESEARCH ARTICLE

Bat coronavirus was detected positive from insectivorous bats in Krau Wildlife Reserve Forest

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ABSTRACT

Bats are flying mammals with unique immune systems that allow them to hold many pathogens. Hence, they are recognised as the reservoir of many zoonotic pathogens. In this study, we performed molecular detection to detect *coronaviruses*, *paramyxoviruses*, *pteropine orthoreoviruses* and *dengue viruses* from samples collected from insectivorous bats in Krau Reserve Forest. One faecal sample from *Rhinolophus* spp. was detected positive for coronavirus. Based on BLASTN, phylogenetic analysis and pairwise alignment-based sequence identity calculation, the detected bat coronavirus is most likely to be a bat betacoronavirus lineage slightly different from coronavirus from China, Philippines, Thailand and Luxembourg. In summary, continuous surveillance of bat virome should be encouraged, as Krau Reserve Forest reported a wide spectrum of biodiversity of insectivorous and fruit bats. Moreover, the usage of primers for the broad detection of viruses should be reconsidered because geographical variations might possibly affect the sensitivity of primers in a molecular approach.

Keywords: Bat diversity; Coronavirus; Paramyxovirus; Pteropine orthoreovirus; Dengue virus.

INTRODUCTION

Bats are a group of mammals under the order Chiroptera characterised by their unique ability of self-driven flight and other odd mammalian adaptations. There are more than 1300 bat species living around the world except in extreme regions within the Antarctic circle (Teeling *et al*., 2018; Banerjee *et al*., 2020). Among the unique adaptations, their immune system is the most outstanding and with great research interest (Teeling *et al*., 2018; Banerjee *et al*., 2020). Bats have evolved antiviral immunity which allows them to tolerate and resist many different viruses. Hence, they have been recognised as the vector or reservoir of many zoonotic pathogens, especially viruses (Banerjee *et al*., 2020). Previously, many medically significant viruses and viruses of concern have been identified in different kinds of bats, such as *lyssaviruses*, *paramyxoviruses*, *coronaviruses*, *filoviruses*, *flaviviruses* and *reoviruses* (Shi, 2013; Letko *et al*., 2020; Siew *et al*., 2023; Wang *et al*., 2023).

However, eliminating bats is not a solution to prevent the outbreak of bat-borne disease because bats have significant ecological and economic importance to humans and the whole ecosystem (Boyles *et al*., 2011; Maine & Boyles, 2015; Biswas *et al*., 2020; Sakoui *et al*., 2020). Thus, in this study, we aimed to perform molecular detection and identification of viruses in insectivorous bats in Krau Wildlife Reserve (KWR) Forest.

Briefly, KWR is an important protected area located in Pahang, with many projects and studies being carried out. This protected reserve area is nearly 63.5 thousands hectares and serve as a lowland tropical rainforest habitat for animals including bats (Kingston *et al*., 2006; Ahmad *et al*., 2018). Furthermore, most surveillance and prevalence studies, especially for coronaviruses, were carried out in East Malaysia (Malaysian Borneo) but not in the West Malaysia (Peninsular Malaysia) (Tan *et al*., 2021; Tan *et al*., 2023; Habeebur-Rahman *et al*., 2023). Hence, KWR was chosen as the site of sample collection.

MATERIALS AND METHODS

Specimen collection

A total of 146 bats were captured and 292 samples (1 oral and 1 faecal sample per bat) were collected in the Lata Bujang collection site (coordinate X:466809, Y:421565), KWR, Pahang in 2019 using harp traps under the Krau Reserve Forest Wildlife Inventory Program. All viable bats were unharmed and released back to the wild after sample collection. The captured bats were morphologically identified based on the Bats of Krau Wildlife Reserve handbook (Kingston *et al*., 2006) and Flaquer *et al*. (2007). Then, oral sampling was performed using an oral swab before the bats were kept in a ventilated bag. After 3 hours, the faeces in each bag were pooled into 2 ml of viral transport medium (VTM, Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera, France) supplemented with 10% fetal bovine serum (FBS) (Biosera, France) at pH 7.4 and 1% penicillin/streptomycin/amphotericin B antibiotics (Abcam, UK)) based on each bat species. The VTM containing bat samples were kept at -80°C and subjected to virus isolation later.

This study was approved by the Department of Wildlife and National Parks Peninsular Malaysia, Ministry of Natural Resources, Environment and Climate Change (JPHL&TN (IP): 100-34/1.24 Jld 17 (26)) and the IMU Joint Committee of Research and Ethics (BP I/2021(05)).

Virus detection and identification

Virus detection was performed using a reverse transcription polymerase chain reaction (RT-PCR) assay. The molecular approach in this study focused on the detection of *coronaviruses* (CoV), *paramyxoviruses* (PMV), *pteropine orthoreoviruses* (PRV) and *dengue viruses* (DenV) only. Viral nucleic acid extraction was performed for both oral and faecal samples using a Viral Nucleic Acid Extraction Kit II (Geneaid Biotech, Taiwan) adhering strictly to the manufacturer's protocol. Reverse transcription was performed using SuperScript III Reverse transcriptase (Invitrogen, USA). Then, PCR amplification was performed with primers adopted from previous studies and was summarised in Table 1. The RT-PCR protocol was adapted with minor modifications from Poon & Peiris (2008). Briefly, heat activation was performed at 94°C for 10 minutes, 40 thermal cycles of denaturing, annealing and extension steps were performed at 94°C (30 seconds), 48°C (30 seconds) and 72°C (40 seconds), respectively. Lastly, the final extension step was performed at 72°C for 5 minutes. The extracted viral genetic materials of RT-PCR positive sample were sent to Apical Scientific (Selangor, Malaysia) for next-generation sequencing services.

Virus identification was performed using Basic Local Alignment Search Tool (BLAST) and phylogenetic analysis. Generally, identified gene sequences were arranged and Multiple Sequence Comparison by Log-Expectation (MUSCLE) aligned using BioEdit (Hall, 1999; Tippmann, 2004) and Molecular Evolutionary Genetics Analysis (MEGA) 11 software, respectively. Reference sequences were identified using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and retrieved from GenBank (accessed on 16th August 2023). Details of the reference sequences are included in Table 2. The best nucleotide model was determined and the maximum likelihood method was performed to construct the phylogenetic tree based on the Tamura-three-parameter model implemented in MEGA 11 (Tamura *et al*., 2021). Bootstrap values were obtained from a random sampling of 1000 replications. Furthermore, a pairwise alignment-based sequence identity calculation was conducted to identify closely related members of the virus using the MUSCLE alignment algorithm in Sequence Demarcation Tool version 1.2 (SDTv1.2) (Edgar, 2004; Muhire *et al*., 2014).

Cell culture and virus isolation

A549 (ATCC CCL-185), Hek293 (ATCC CRL-1573), Vero (ATCC CCL-81) and C6/36 (ATCC CRL-1660) cells were used to isolate unknown viruses from bat samples. C6/36 cells were a kind gift from Dr. Chua Kaw Bing. A549, Hek293 and Vero cell cultures were incubated at 37°C, 5% carbon dioxide (CO_2) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS and 1% penicillin/streptomycin/amphotericin B antibiotics. C6/36 cells were incubated at 28°C, 5% CO_2 in RPMI 1640 supplemented with 5% FBS and 1% penicillin/streptomycin/amphotericin B antibiotics. Each cell was seeded into a T25 tissue culture flask at a cell confluency of approximately 25% and added with 200µL of the collected samples. Microscopic examination for the presence of cytopathic effect (CPE) was performed daily under an inverted microscope. The virus isolation protocol was adapted from a previous study with some modifications (Chua *et al*., 2001).

RESULTS

Bat Biodiversity

Morphological identification (Figure 1, some bat species are not included) revealed a total of 146 bats belonging to at least 18 species were captured in KWR, Pahang. Referring to Figure 2, the majority of bats from KWR belonged to the *Rhinolophidae* (33.6%) family, followed by *Hipposideridae* (including *Coelops* spp., 32.9%), *Vespertilionidae* (including *Murina* spp., *Kerivoula* spp., *Tylonycteris* spp., and *Myotis* spp., 18.5%), *Molossidae* (*Mops mops*, 11%), *Nycteridae* (*Nycteris tragata*, 2%) and *Pteropodidae* (including *Cynopterus* spp., *Balionycteris maculate*, 2%) families. Interestingly, one of the bats captured in KWR, *Coelops* spp., was a rare species from the *Hipposideridae* family (figure not shown).

Virus detection and identification

All samples were tested negative for CoV, PMV, PRV and DenV using RT-PCR detection. Except for one *Rhinolophus* spp. faecal sample was tested positive using CoV primers. Similarly, no virus was isolated from the sample co-culture, including the sample that tested positive for CoV. The positive sample (4039858_KA367_F4) had been blindly passaged in A549, Hek293, Vero and C6/36 cells but no cytopathic effect was observed after 6 passages. The RNA-dependent RNA polymerase (RDRP) gene of the bat CoV 4039858_KA367_F4 (Accession: OR485299, 408 base-pair) was sequenced and was compared through BLASTN and phylogenetic analysis (Figure 3). Results obtained from BLASTN indicated that the bat CoV 4039858_KA367_F4 is the closest to bat betacoronavirus. Lastly, the pairwise alignment-based sequence identity calculation (Figure 4) indicated that the bat CoV 4039858_KA367_F4 has a very high percentage (nearly 83%) of pairwise identity when compared with bat betacoronavirus isolate Beta/BatG/SwkMY/G42/2021 (Accession: MZ574070.1, Malaysia), coronavirus PREDICT CoV-51 PREDICT_CoV-51/PSW 00163 (Accession: KX284939.1, Malaysia) and Hipposideros bat coronavirus isolate PREDICT-CoV-51/PDF-0663 (Accession: MZ293757.1, Malaysia), but not with human CoV 229E (Accession: MT797634.1, Hong Kong), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Wuhan-Hu-1 isolate (Accession: MN908947.3, China) and human CoV HKU1 (Accession: NC_006577.2, Hong Kong).

DISCUSSION

KWR is a reserved forest under the protection of the government and Malaysia's National Forestry Act (1984), and it is known to support a more diverse bat population (Kingston *et al*., 2006; Ahmad *et al*., 2018). According to Kingston *et al*. (2006), about 50% of the insectivorous bats in Peninsular Malaysia are from the family *Vespertilionidae*. However, the most abundant bat species in KWR are from the *Rhinolophidae* family. Few possible reasons may include: (1) the time of bat trapping and sample collection; (2) the habitat and environment of sample collection site. Firstly, bats from *Vespertilionidae* family, also known as evening bats or vesper bats, are usually active in the evening (Kingston *et al*., 2006). Thus, the traps may not catch a lot vesper bats if they were set up around late afternoon to early evening (around 5pm to 7pm). Secondly, perhaps the sample collection sites were the roosting area to other bat family instead of *Vespertilionidae* family.

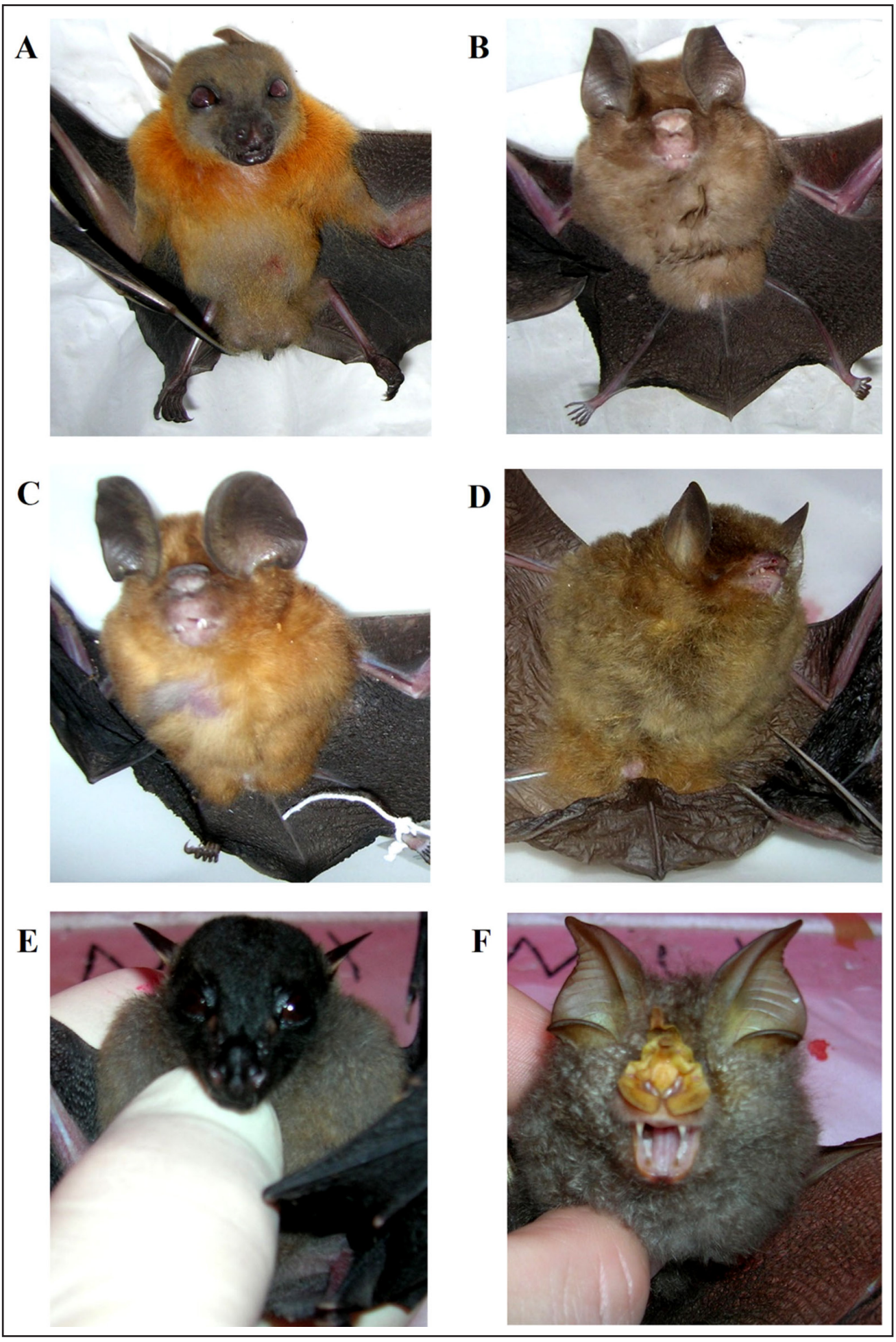
Table 1. Primer sequences for *coronaviruses*, *paramyxoviruses*, *pteropine orthoreoviruses* and *dengue viruses*

Target Viruses	Primer	Sequences (5'-3')	Citation
Coronaviruses	G1BtCovF1 G1BtCovR1	GGTTGGGACTATCCTAAGTGTGA CCATCATCAGATAGAATCATCAT	(Poon & Peiris, 2008)
Paramyxoviruses	PAR-F1 PAR-F2 PAR-R	GAAGGITATTGTCAIAARNTNTGGAC GTTGCTTCAATGGTTCARGGNGAYAA GCTGAAGTTACIGGITCICCDATRTTNC	(Tong *et al*., 2008)
Pteropine orthoreoviruses	Sig1F Sig1R	GTGCCGTGTTCGACTTCTTTAC ACAACAGCATTCGACCCTAC	(Tee *et al*., 2023)
Dengue viruses	D1 D2	CAATATGCTGAAACGCGCGAGAAACCG TTGCACCAACAGTCAATGTCTTCAGGTTC	(Teo *et al*., 2017)
DenV-1	Dcon TS1	AGTTGTTAGTCTACGTGGACCGACA CGTCTCAGTGATCCGGGGG	
DenV-2	Dcon TS2	AGTTGTTAGTCTACGTGGACCGACA CGCCACAAGGGCCATGAACAG	
DenV-3	D1 TS3	TCAATATGCTGAAACGCGCGAGAAACCG TAACATCATCATGAGACAGAGC	
DenV-4	D1 TS4	TCAATATGCTGAAACGCGCGAGAAACCG CTCTGTTGTCTTAAACAAGAGA	

Table 2. Details of Reference Sequences

No.	Name of Reference Sequence	GenBank Accession Number	Origin	Genus
1	Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome	MN908947.3	Wuhan, China	Betacoronavirus
2	Human coronavirus 229E isolate HK2042, complete genome	MT797634.1	Hong Kong, China	Alphacoronavirus
3	Human coronavirus HKU1, complete genome	NC_006577.2	Hong Kong, China	Betacoronavirus
4	Bat betacoronavirus isolate R1B8 RNAdependent RNA polymerase (RdRp)gene, partial cds	MT467451.1	Philippines	Betacoronavirus
5	Bat coronavirusHipposideros/KT/Thailand/2007 isolateKT nonstructural protein-like gene, partial sequence	HM017067.1	Thailand	Betacoronavirus
6	Severe acute respiratory syndromerelated coronavirus isolate BtCoV/Rhinolophusferrumequinum/LUX/LUX16_A_24/2016 RNA-dependent RNA polymerase gene, partial cds	KY502395.1	Luxembourg	Betacoronavirus
7	Bat betacoronavirus isolateBeta/BatG/SwkMY/G42/2021 RNAdependent RNA polymerase gene, partialcds	MZ574070.1	Malaysia	Betacoronavirus
8	Coronavirus PREDICT CoV-51PREDICT_CoV-51/PSW 00163 RNAdependent RNA polymerase mRNA, partial cds	KX284939.1	Malaysia	Betacoronavirus
9	Hipposideros bat coronavirus isolate PREDICT-CoV-51/PDF-0663, complete genome	MZ293757.1	Malaysia	Betacoronavirus

The bat CoV 4039858_KA367_F4 detected in *Rhinolophus* spp. is most likely to be a type of bat betacoronavirus that highly similar to bat betacoronavirus isolate Beta/BatG/SwkMY/G42/2021, coronavirus PREDICT CoV-51 PREDICT_CoV-51/PSW 00163 and Hipposideros bat coronavirus isolate PREDICT-CoV-51/PDF-0663. When grouping coronavirus into one of the four genera, only a virus with above 90% amino acid sequence identity in the conserved key domains of RDRP, also known as a replicase-transcriptase polyprotein, will be grouped under the same genus (Howley *et al*., 2020). In this study, none of the reference sequences has a minimum of 90% amino acid sequence identity in RDRP when compared with bat CoV 4039858_KA367_F4. Thus, we suggested that this bat CoV in Pahang, Malaysia might be a distinct lineage of bat CoV which is different from the CoV in China, Philippines, Thailand and Luxembourg (Figure 3). Therefore, the bat CoV 4039858_KA367_F4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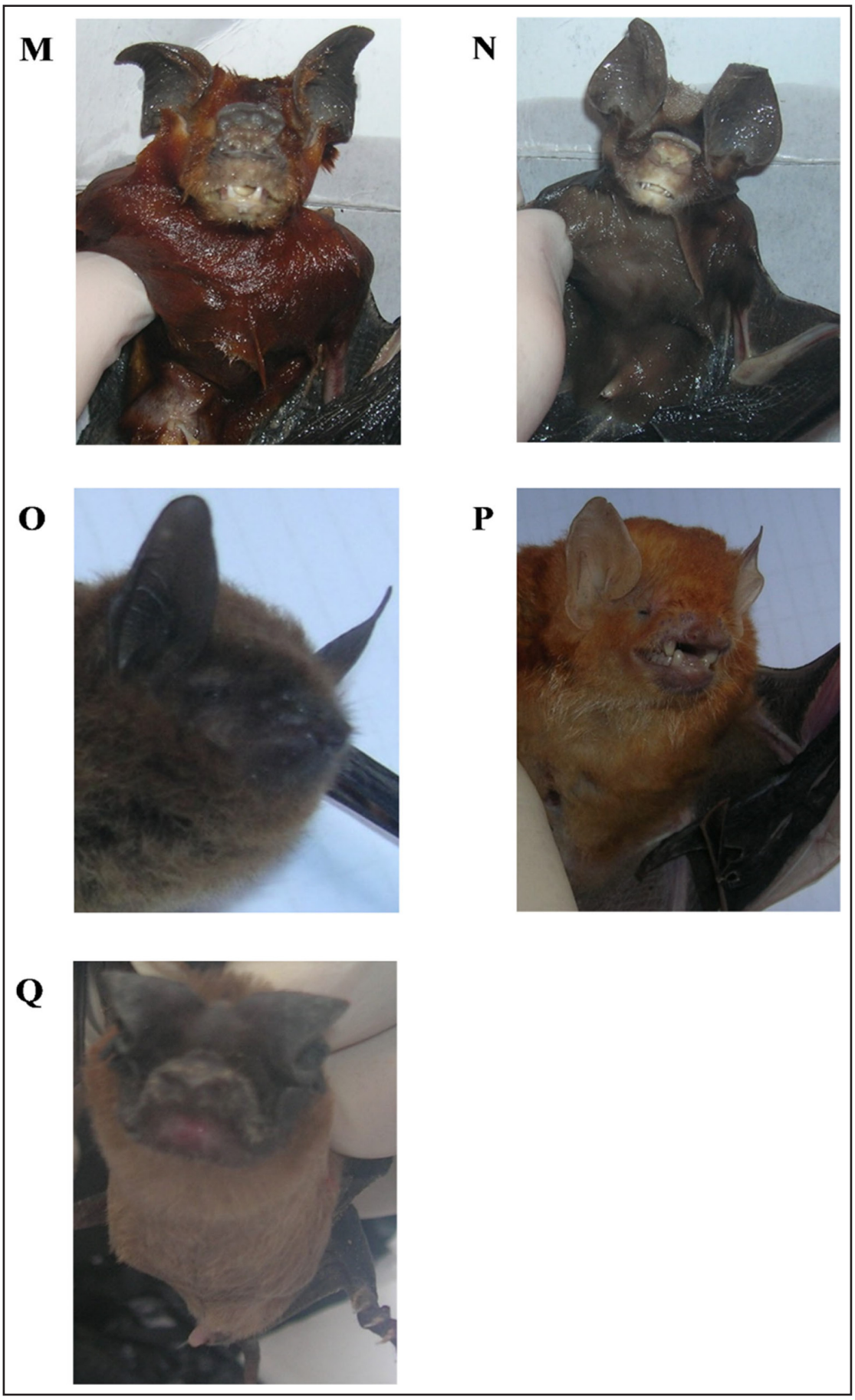

Figure 1. The external morphology of different bats. (A) *Cynopterus brachyotis*; (B) *Hipposideros cervinus*; (C) *Hipposideros cineraceus*; (D) *Kerivoula pellucida*; (E) *Balionycteris maculata*; (F) *Rhinolophus trifoliatus*; (G) *Tylonycteris robustula*; (H) *Rhinolophus affinis*; (I) *Nycteris tragata*; (J) *Kerivoula papillosa*; (K) *Hipposideros larvatus*; (L) *Rhinolophus trifoliatus*; (M) *Hipposideros armiger*; (N) *Hipposideros* spp.; (O) *Myotis* spp.; (P) *Myotis hermani*; (Q) *Mops mops*.

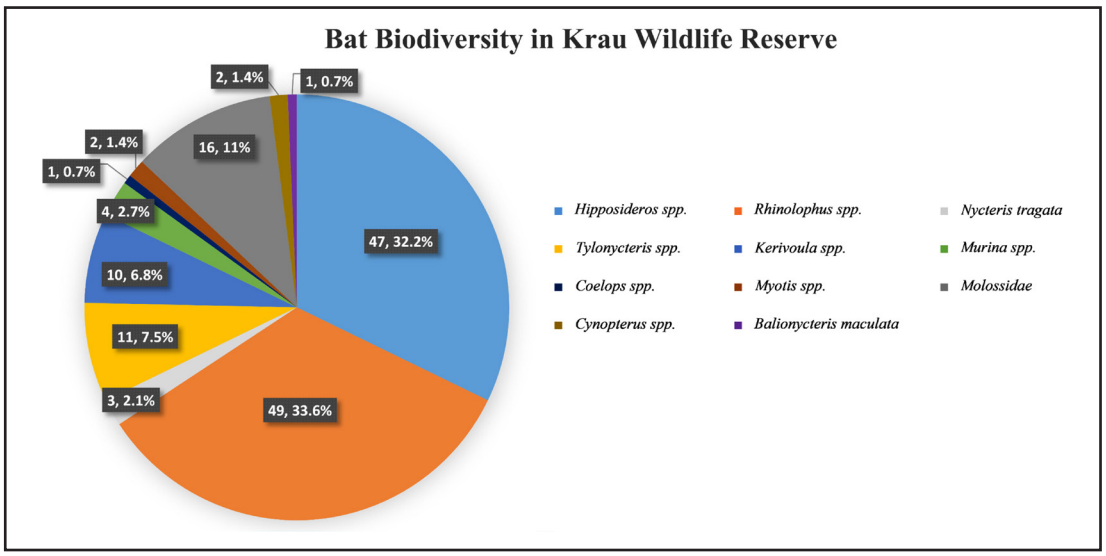

Figure 2. The bat biodiversity in Krau Wildlife Reserve, Pahang, Malaysia.

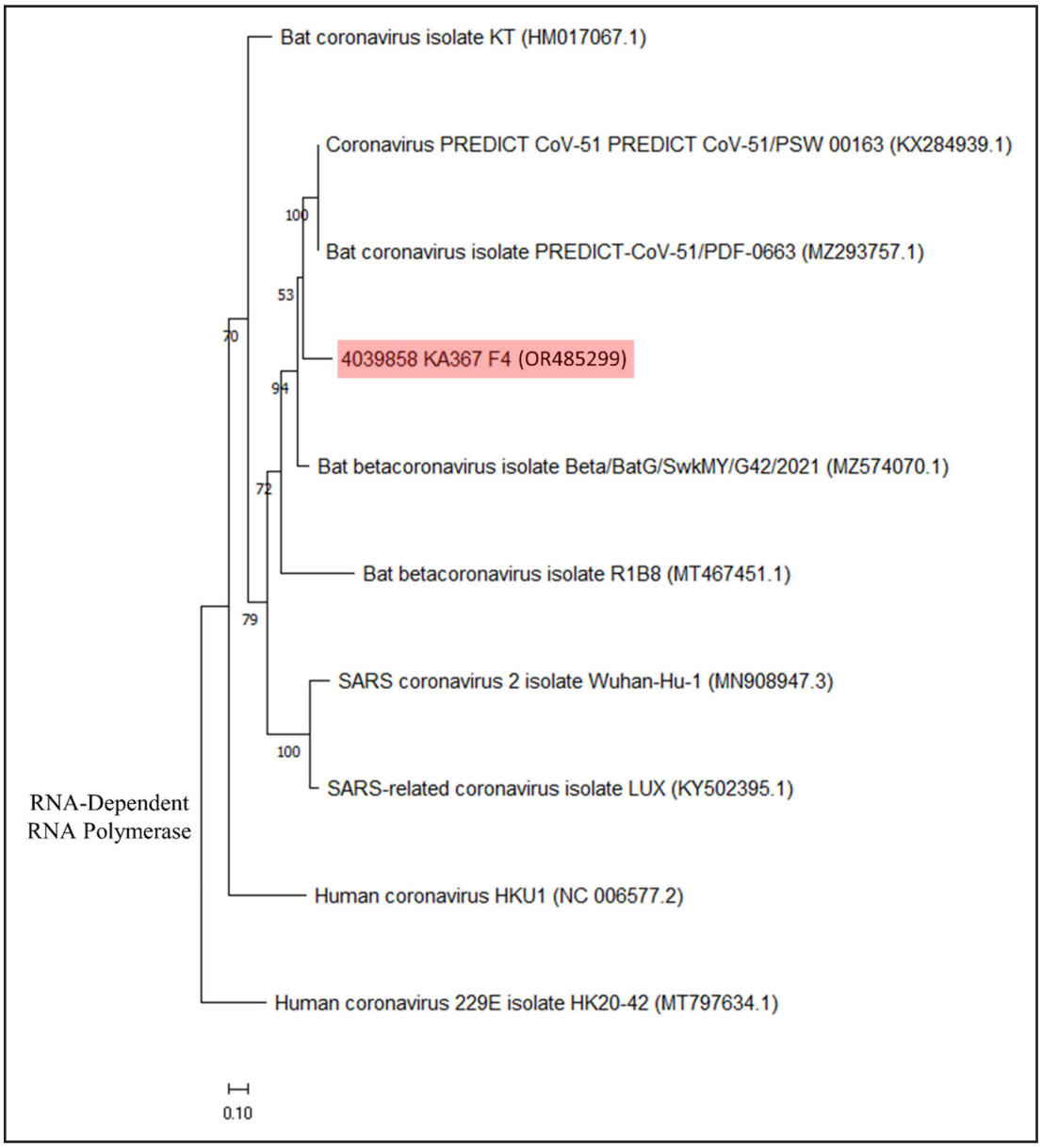

Figure 3. The phylogenetic tree of *Rhinolophus* spp. bat coronavirus isolate 4039858_KA367_F4 (highlighted in pinkish-red) and reference sequences using RNA-dependent RNA polymerase gene sequence. The phylogenetic tree was constructed based on the Tamura-3-parameter model.

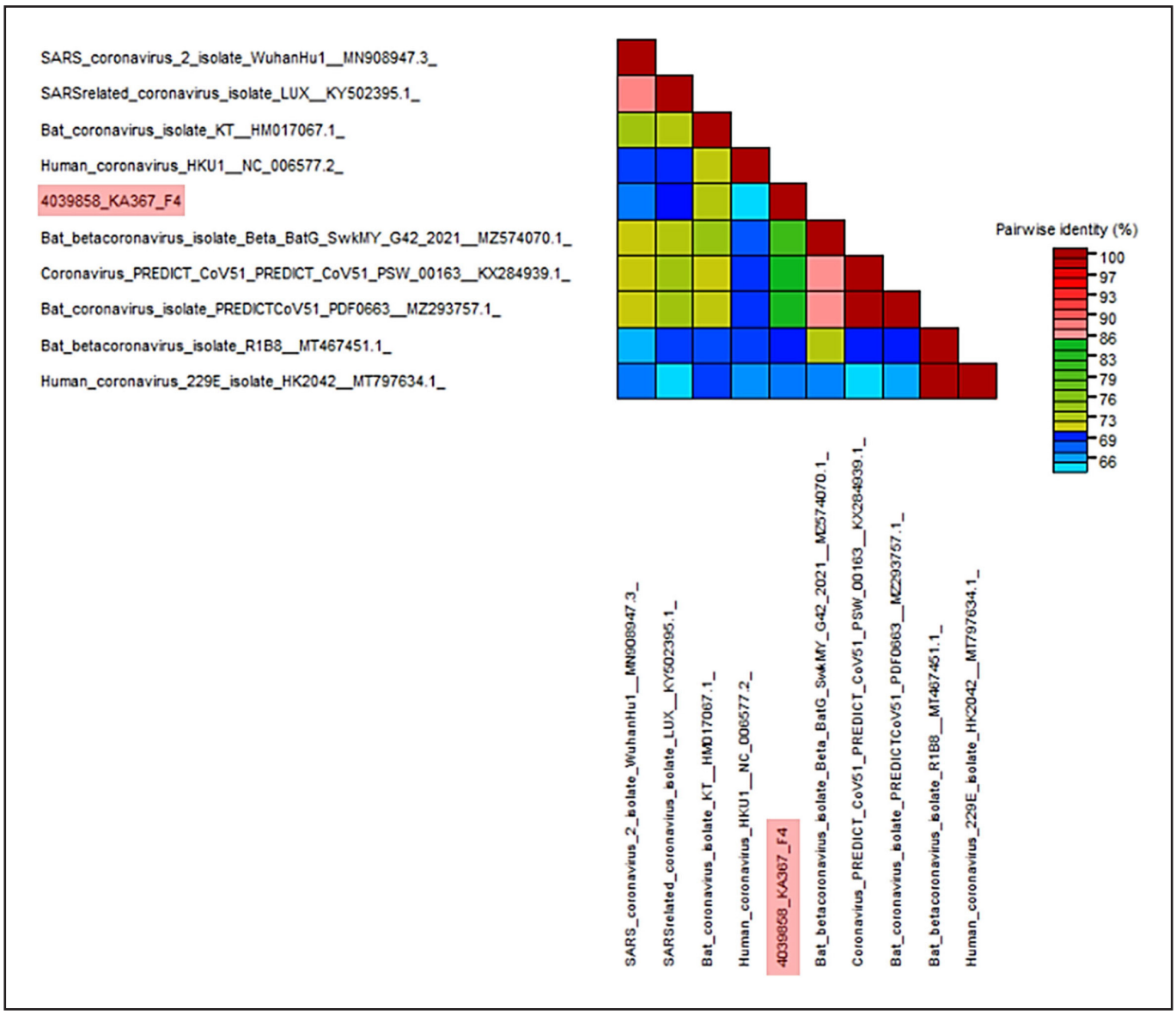

Figure 4. The pairwise alignment-based sequence identity calculation of *Rhinolophus* spp. bat coronavirus 4039858_KA367_F4 (highlighted in pinkish-red, accession: OR485299.1) and reference sequences using RNA-dependent RNA polymerase gene sequence. The bat CoV 4039858_KA367_F4 has a very high percentage of pairwise identity when compared with three bat betacoronaviruses (Accession: MZ574070.1, KX284939.1 and MZ293757.1), but not with human CoV 229E, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) WuhanHu-1 isolate and human CoV HKU1.

was an unclassified coronavirinae, we should also reconsider the usage and sensitivity of the CoV primers adopted from other studies.

The limitations in this study are: (1) No virus was successfully isolated; (2) only a partial RDRP sequence was obtained in this study, instead of the complete genome (the sample was not enough to proceed for full genome sequencing); (3) Seroprevalence study was not included in this study.

In conclusion, the majority of bats from KWR belonged to the *Rhinolophidae* (33.6%) family, followed by *Hipposideridae* (including *Coelops* spp., 32.9%), *Vespertilionidae* (including *Murina* spp., *Kerivoula* spp., *Tylonycteris* spp., and *Myotis* spp., 18.5%), *Molossidae* (*Mops mops*, 11%), *Nycteridae* (*Nycteris tragata*, 2%) and *Pteropodidae* (including *Cynopterus spp*., *Balionycteris maculate*, 2%) families. Furthermore, this study demonstrated that low prevalence of bat coronaviruses detected from insectivorous and fruit bats in Malaysia. Nevertheless, continuous surveillance of bat virome should be encouraged.

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Disclosure statement

The authors declare no conflict of interest. In addition, funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the article, or in the decision to publish the results.

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