

Host range analysis of Rousettus Bat Coronavirus HKU-9

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ABSTRACT

The representative member of the genus Nobecovirus, Bat Coronavirus HKU-9, was one of the least known members of the genus Betacoronaviruses. Here we performed a detailed analysis of the known and potential host ranges of HKU-9.

METHODS

Keyword Search

In order to obtain all strains of HKU9 available on GenBank, we performed a Keyword Search on GenBank with the Keyword HKU9.

Table 1: Result of all HKU9 strains returned on Keyword search

Accession	Host
JN857318.1	bat feces*
JN857317.1	bat feces*
JN857316.1	bat feces*
JN857315.1	bat feces*
JN857314.1	bat feces*
JN857313.1	bat feces*
JN857312.1	bat feces*
JN857311.1	bat feces*
MN312670.1	Eonycteris spelaea
MN312669.1	Eonycteris spelaea
MN312668.1	Eonycteris spelaea
MN312631.1	Rousettus sp.
MN312630.1	Rousettus sp.
MN312629.1	Rousettus sp.
MN312628.1	Rousettus sp.
MN312627.1	Rousettus sp.
MN312626.1	Unknown**
MN312624.1	Rousettus sp.
MN312621.1	Rousettus sp.
MN312620.1	Rousettus sp.
MN312619.1	Eonycteris spelaea

MN312618.1	Rousettus sp.
MN312617.1	Rousettus sp.
MN312616.1	Eonycteris spelaea
MN312615.1	Rousettus sp.
MN312614.1	Rousettus sp.
MN312610.1	Rousettus sp.
KX286294.1	Rousettus sp.
KX286293.1	Rousettus sp.
KX286259.1	Rousettus aegyptiacus
KX285761.1	Macroglossus sp.
KX285758.1	Macroglossus sp.
KX285425.1	Rousettus aegyptiacus
KX285424.1	Rousettus aegyptiacus
KX285066.1	Rousettus sp.
KX285065.1	Rousettus sp.
KX284961.1	Eidolon helvum
KX284959.1	Rousettus aegyptiacus
KX284938.1	Rousettus sp.
KX284935.1	Rousettus sp.
KX284933.1	Rousettus sp.
KX284932.1	Eonycteris spelaea
KX284931.1	Rousettus sp.
KX284925.1	Rousettus sp.
KX284924.1	Rousettus sp.
KX284920.1	Rousettus sp.
KX284919.1	Eonycteris spelaea
KX284917.1	Rousettus sp.
KX284915.1	Rousettus sp.
KX284913.1	Rousettus amplexicaudatus
KX284912.1	Eonycteris spelaea
KX284911.1	Eonycteris spelaea
KX284910.1	Rousettus amplexicaudatus
KX284909.1	Rousettus leschenaultii
KX284908.1	Eonycteris spelaea
KX284907.1	Rousettus sp.
KX284905.1	Rousettus sp.
KX284904.1	Rousettus sp.
KX284903.1	Rousettus sp.
KX284902.1	Rousettus sp.
KY010637.1	Eonycteris spelaea
KY010636.1	Eonycteris spelaea
KY010635.1	Rousettus amplexicaudatus
KY010634.1	Rousettus leschenaultii

KY010633.1	Eonycteris spelaea
KY010632.1	Rousettus sp.
KY010631.1	Rousettus sp.
KY010630.1	Rousettus sp.
KY010629.1	Rousettus sp.
MG762674.1	Rousettus sp.
MG762673.1	Rousettus leschenaulti
MG762672.1	Rousettus leschenaulti
MG762671.1	Rousettus leschenaulti
MG762670.1	Rousettus leschenaulti
MG762669.1	Rousettus sp.
MG762668.1	Rousettus sp.
MG762667.1	Rousettus leschenaulti
MG762666.1	Rousettus leschenaulti
MG762665.1	Rousettus sp.
MG762664.1	Rousettus sp.
MG762663.1	Rousettus sp.
MG762662.1	Rousettus sp.
MG762661.1	Rousettus sp.
MG762660.1	Rousettus sp.
MG762659.1	Rousettus sp.
MG762658.1	Rousettus leschenaulti
MG762657.1	Rousettus leschenaulti
MG762656.1	Rousettus leschenaulti
MG762655.1	Rousettus leschenaulti
MG762654.1	Rousettus leschenaulti
MG762653.1	Rousettus leschenaulti
MG762652.1	Rousettus leschenaulti
MG762651.1	Rousettus leschenaulti
MG762650.1	Rousettus leschenaulti
MG762649.1	Rousettus leschenaulti
MG762648.1	Rousettus leschenaulti
MG762647.1	Rousettus leschenaulti
MG762646.1	Rousettus leschenaulti
MG762645.1	Rousettus leschenaulti
MG762644.1	Rousettus leschenaulti
MG762643.1	Rousettus leschenaulti
MG762642.1	Rousettus leschenaulti
MG762641.1	Rousettus leschenaulti
MG762640.1	Rousettus leschenaulti
MG762639.1	Rousettus leschenaulti
MG762638.1	Rousettus leschenaulti
MG762637.1	Rousettus leschenaulti

MG762636.1	Rousettus leschenaulti
MG762635.1	Rousettus leschenaulti
MG762634.1	Rousettus leschenaulti
MG762633.1	Rousettus leschenaulti
MG762632.1	Rousettus leschenaulti
MG762631.1	Rousettus leschenaulti
MG762630.1	Rousettus leschenaulti
MG762629.1	Rousettus leschenaulti
MG762628.1	Rousettus leschenaulti
MG762627.1	Rousettus leschenaulti
MG762626.1	Rousettus leschenaulti
MG762625.1	Rousettus sp.
MG762624.1	Rousettus sp.
MG762623.1	Rousettus sp.
MG762622.1	Rousettus sp.
MG762621.1	Rousettus sp.
MG762620.1	Rousettus sp.
MG762619.1	Rousettus sp.
NC_009021.1	Bat***
HM211101.1	bat BF_506 ****
MT337384.1	Rousettus sp.
EF065514.1	Bat***
EF065515.1	Bat***
EF065516.1	Bat***
MT350584.1	Rousettus
MT350585.1	Rousettus
HM211099.1	bat BF_258 ****
HM211098.1	bat BF_258 ****
EF065513.1	Bat***
MT350598.1	Eonycteris spelaea
NC_030886.1 +	Rousettus leschenaulti
KU762337.1 +	Rousettus leschenaulti
KU762338.1 +	Rousettus leschenaulti
KX652398.1 +	Rousettus leschenaulti
KX652399.1 +	Rousettus leschenaulti
KX652400.1 +	Rousettus leschenaulti
KX652401.1 +	Rousettus leschenaulti
KX652402.1 +	Rousettus leschenaulti
KX652397.1 +	Rousettus leschenaulti
KX652396.1 +	Rousettus leschenaulti
KX652395.1 +	Rousettus leschenaulti
KX652394.1 +	Rousettus leschenaulti
KX652393.1 +	Rousettus leschenaulti

KX652392.1 +	Rousettus leschenaulti
KX652391.1 +	Rousettus leschenaulti
KX652390.1 +	Rousettus leschenaulti
KX652389.1 +	Rousettus leschenaulti
KX652388.1 +	Rousettus leschenaulti
KX652387.1 +	Rousettus leschenaulti
KX652386.1 +	Rousettus leschenaulti
KX652385.1 +	Rousettus leschenaulti
KX652384.1 +	Rousettus leschenaulti
KX652383.1 +	Rousettus leschenaulti
KX652382.1 +	Rousettus leschenaulti
KX652381.1 +	Rousettus leschenaulti
KX652380.1 +	Rousettus leschenaulti
KX652379.1 +	Rousettus leschenaulti
MN312667.1 +	Eonycteris spelaea
MN312623.1 +	Eonycteris spelaea
MN312622.1 +	Eonycteris spelaea
MN312613.1 +	Eonycteris spelaea
MN312612.1 +	Eonycteris spelaea
MN312611.1 +	Unknown*****
MG762692.1 +	Eonycteris spelaea
MG762691.1 +	Eonycteris spelaea
MG762690.1 +	Eonycteris spelaea
MG762689.1 +	Eonycteris spelaea
MG762688.1 +	Eonycteris spelaea
MG762687.1 +	Eonycteris spelaea
MG762686.1 +	Eonycteris spelaea
MG762685.1 +	Eonycteris spelaea
MG762684.1 +	Eonycteris spelaea
MG762683.1 +	Eonycteris spelaea
MG762682.1 +	Eonycteris spelaea
MG762681.1 +	Eonycteris spelaea
MG762680.1 +	Eonycteris spelaea
MG762679.1 +	Eonycteris spelaea
MG762678.1 +	Eonycteris spelaea
MG762677.1 +	Eonycteris spelaea
MG762676.1 +	Eonycteris spelaea
MG762675.1 +	Eonycteris spelaea
MG762618.1 +	Eonycteris spelaea
MG762617.1 +	Eonycteris spelaea
MG762616.1 +	Eonycteris spelaea
MG762615.1 +	Eonycteris spelaea
MG762614.1 +	Eonycteris spelaea

MG762613.1 +	Eonycteris spelaea
MG762612.1 +	Eonycteris spelaea
MG762611.1 +	Eonycteris spelaea
KU182982.1 +	Rousettus leschenaulti
MG762610.1 +	Eonycteris spelaea
MG762609.1 +	Eonycteris spelaea
MG762608.1 +	Eonycteris spelaea
MG762607.1 +	Eonycteris spelaea
MG762606.1 +	Eonycteris spelaea

*:From SRA: DRA000500

** : is identical to MN312614.1

*** identified as *Rousettus lechenaulti*. Culture failed. PMC: 1797546

**** identified as *Rousettus leschenaulti* Not cultured. PMC: 2953156

***** most related to KU762337.1. All full-length coverage on BLAST have host Rousettus leschenaulti or Eonycteris spelaea

+ GCCDC1 genomes.

RESULTS

Host range determination of HKU9

In order to deduce the nature of DRA000500, a SRA TRACE analysis is conducted.

Viral metagenomic Analysis of bat fecal samples (DRR001793)

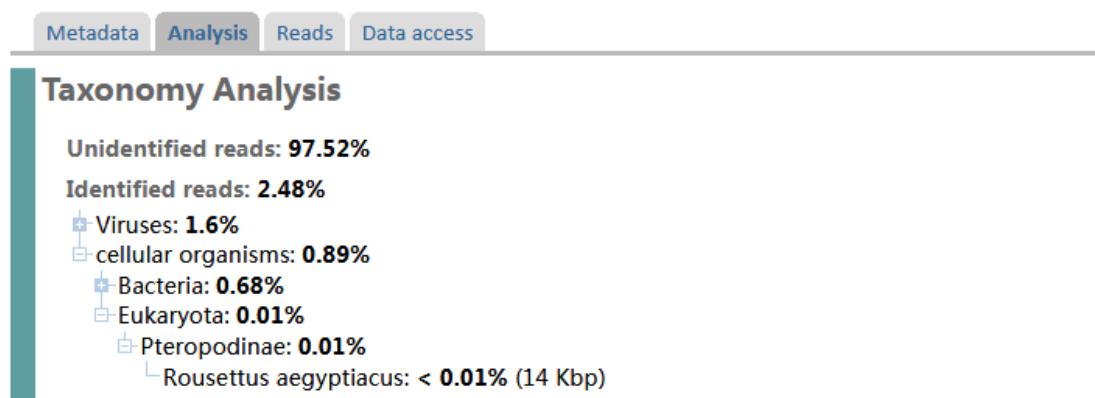


Fig.1: TRACE result of DRA000500

The TRACE result suggest a Fecal sample dominated by bacteria, and the only discernible bat reads were 31/14Kbp reads from the Pteropodinae reference species Rousettus Aegyptiacus. In addition, all eukaryotic reads TRACES to Pteropodinae/Rousettus—indicating a sample of Pteropodinae (Megabat) in origin.

This origin is confirmed by BLAST search result covering a large proportion of the Rousettus leschenaulti reference Mitochondrion. [NC_046927.1](https://www.ncbi.nlm.nih.gov/nuccore/NC_046927.1).

ref|NC_046927.1|

Rousettus leschenaultii isolate CKM109 mitochondrion, complete genome

Distribution of the top 119 Blast Hits on 119 subject sequences

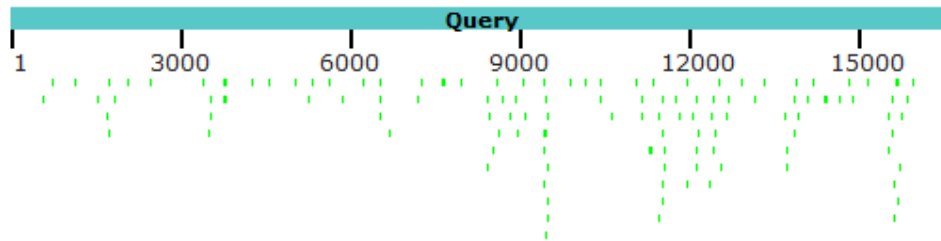


Fig.2: BLAST result of DRA000500.

The "host" section of all HKU9 samples are divided in the following sections:

14 sequences are listed as from *Eonycteris spelaea*.

46 sequences are listed as from *Rousettus* sp

49 sequences are listed as from *Rousettus leschenaultii*

4 sequences are listed as from *Rousettus aegyptiacus*

1 sequence is listed as from *Eidolon helvum*

3 sequences are listed as from *Rousettus amplexicaudatus*

2 sequences are listed as from *Macroglossus* sp.

8 sequences are listed as from "bat feces" (DRA000500)

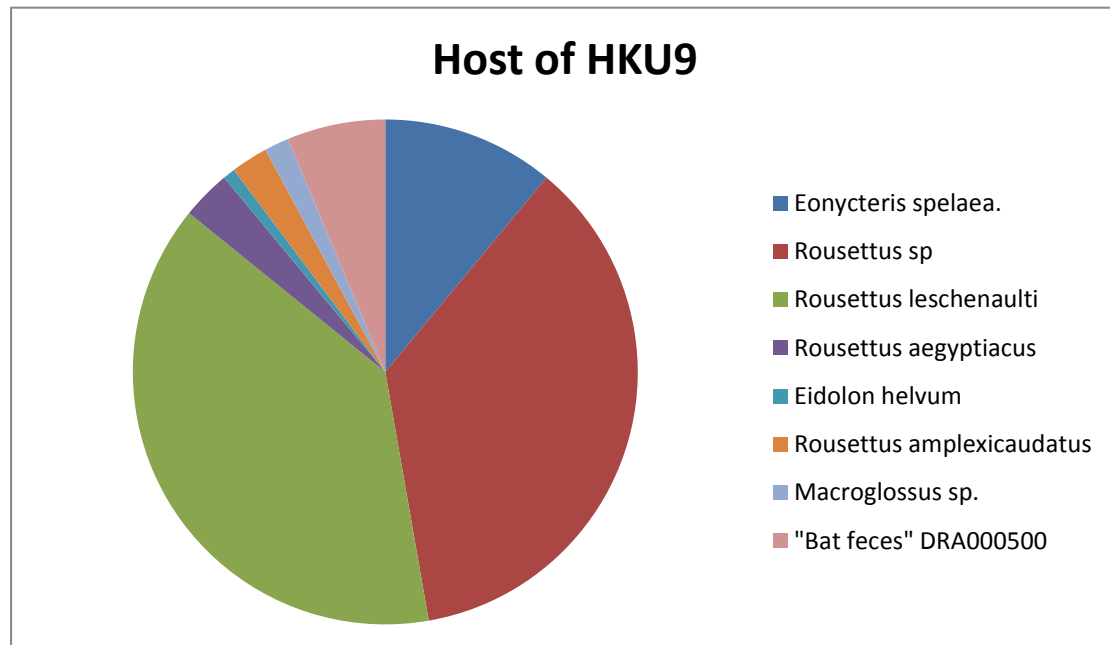


Fig.3: Chart of HKU9 Host range.

Host range determination of GCCDC1

The “host” section of all GCCDC1 samples are divided in the following sections:

28 sequences are listed as from *Rousettus leschenaulti*

36 sequences are listed as from *Eonycteris spelaea*

1 sequence is listed as unknown.

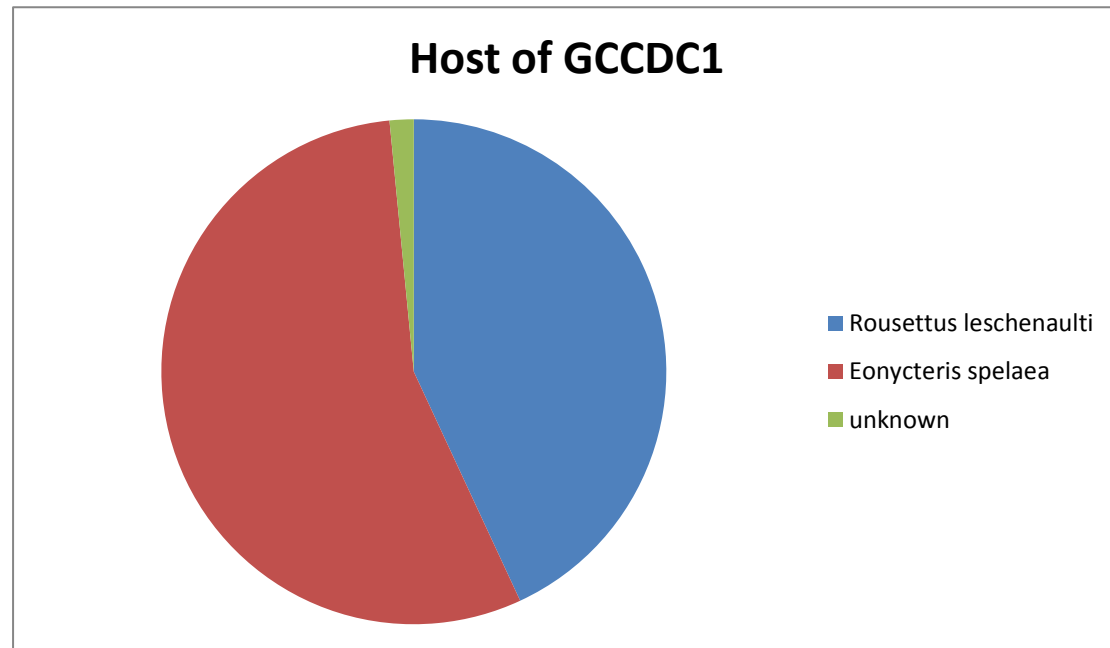


Fig.4: Chart of GCCDC1 Host range.

Phylogenetic analysis of Host species.

The phylogenetic analysis of the bat species obtained are determined using Keyword search on the respective species.

Eonycteris spelaea<- [Eonycteris](#)<- [Macroglossinae](#)<- [Pteropodidae](#)

Rousettus sp<- [Pteropodidae](#)

Rousettus leschenaulti<- [Rousettus](#)<- [Pteropodidae](#)

Rousettus aegyptiacus<- [Rousettus](#)<- [Pteropodidae](#)

Eidolon helvum< [Eidolon](#)<- [Pteropodidae](#)

Rousettus amplexicaudatus<- [Rousettus](#)<- [Pteropodidae](#)

Macroglossus sp.<- [Macroglossinae](#)<-[Pteropodidae](#)

All known hosts of HKU9 and GCCDC1 belong to Pteropodidae, or fruit bats.

DISCUSSIONS

DRA000500 And bat colonies

Despite the original depositors of JN857311.1 to JN857318.1 Claimed a source of Bat feces from a Hipposideros-dominated bat colony, bioinformatics assays including both SRA Trace analysis and BLAST analysis of the actual NGS dataset of the 8 pooled Fecal samples, DRA000500, Suggest a sample with Roussettus sp being the dominant Eukaryotic species and contained large amount of 100% matched reads on the Mitochondrial DNA. As the first Hipposideros RefSeq is deposited in 31-OCT-2011, this imply the source of bat feces as being at least heavily contaminated by material of Roussettus sp. Origin.

<input checked="" type="checkbox"/>	Roussettus leschenaultii isolate VN431 mitochondrion_complete_genome	65.8	65.8	100%	3e-08	100.00%	MN816355.1
<input checked="" type="checkbox"/>	Roussettus leschenaultii isolate CKM109 mitochondrion_complete_genome	65.8	65.8	100%	3e-08	100.00%	NC_046927.1

Fig.4a Single read BLAST result of the read `gnl|SRA|DRR001793.7141756 FC30UF2AAXX:6:82:958:809 AACCTTGCTAGAAACTCACACCTCTGGATATGGCT` from DRA000500

<input checked="" type="checkbox"/>	Roussettus leschenaultii isolate VN431 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	MN816355.1
<input checked="" type="checkbox"/>	Roussettus leschenaultii isolate CKM109 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	NC_046927.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus voucher MHNG-1971.070 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	MN816351.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate T2209 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	MN816350.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus voucher MHNG-1807.092 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	MN816349.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate T246 mitochondrion_complete_genome	63.9	63.9	97%	1e-07	100.00%	MN816348.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus mitochondrion_partial_genome	63.9	63.9	97%	1e-07	100.00%	KC702803.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_4538 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274462.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_3159 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274461.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_3115 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274460.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_2917 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274459.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_4747 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274458.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_Sen1316 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274457.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_1543 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274456.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_2531 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274455.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus isolate N_2524 NADH dehydrogenase subunit 1 (nd1) gene_partial_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	JX274454.1
<input checked="" type="checkbox"/>	Roussettus leschenaultii isolate A8 NADH dehydrogenase subunit 1 (ND1) gene_complete_cds_mitochondrial	63.9	63.9	97%	1e-07	100.00%	DQ888649.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus mitochondrial DNA_complete_genome	63.9	63.9	97%	1e-07	100.00%	AB205183.1
<input checked="" type="checkbox"/>	Roussettus oblivivus voucher FMNH-220042 mitochondrion_complete_genome	60.2	60.2	91%	1e-06	100.00%	MN816359.1
<input checked="" type="checkbox"/>	Roussettus oblivivus voucher FMNH-220017 mitochondrion_complete_genome	60.2	60.2	91%	1e-06	100.00%	NC_046929.1
<input checked="" type="checkbox"/>	Roussettus aegyptiacus voucher MNHN-ZM-2011-863 mitochondrion_complete_genome	58.4	58.4	97%	5e-06	97.06%	MN816347.1

Fig.4b Single read BLAST result of the read `gnl|SRA|DRR001793.1716180 FC30UF2AAXX:6:19:1175:1582 CGGGGGTGTGGTATGGGTAGGGGGATTACATTGT` from DRA000500

Since they used PCR analysis on the samples to obtain the partial N genes from the samples, The presence of Roussettus spp. Sequences as the only dominant Eukaryotic sequences in the SRA dataset, strongly imply the PCR product being of Roussettus spp. In origin. As no samples of HKU9 with a defined host were obtained from bats other than family Pteropodidae, the presence of large amount of Roussettus spp. Material within the SRA dataset, with 119 BLAST results mapping just to the R. leschenaultii mitochondrial genome, strongly suggest that it was R.leschenaultii, a natural host of HKU9 coronaviruses, that produced the sequences that ultimately generated the Amplicons as seen as JN857311.1 to JN857318.1 from the mixed fecal samples collected by the depositors of JN857311.1 to JN857318.1

Notably, JN857311.1 to JN857318.1 are all amplicons from the mixed samples—NGS itself did not reveal any sequences related to HKU9-like viruses, indicating the original level of HKU9 nucleotides is very low in the fecal samples. As PCR is a very sensitive technique, this makes the HKU9 sequence amplicons in-line with a contaminant rather than a dominant viral strain in the

sample, further supporting its origin from the Rousettus sp. Derived material within the DRA000500 samples.

Determination of potential hosts of HKU9

In order to determine the potential host range of HKU9, a Keyword search of cell culture isolation attempts of HKU9 is conducted

Table 2. Keyword search result on “HKU9” and “Isolation” or “Culture” or “Cell”

Title	Cells used	Result
Middle East respiratory syndrome coronavirus and bat coronavirus HKU9 both can utilize GRP78 for attachment onto host cells[1]	“RLK or other cell lines”	Not successful
Putative Receptor Binding Domain of Bat-Derived Coronavirus HKU9 Spike Protein: Evolution of Betacoronavirus Receptor Binding Motifs[2]	Not attempted.	N/A
Comparative Analysis of Twelve Genomes of Three Novel Group 2c and Group 2d Coronaviruses Reveals Unique Group and Subgroup Features[3]	“LLC-Mk2 (rhesus monkey kidney), MRC-5 (human lung fibroblast), FRhK-4 (rhesus monkey kidney), Huh-7.5 (human hepatoma), Vero E6 (African green monkey kidney), and HRT-18 (colorectal adenocarcinoma)”	Not successful
Replication of MERS and SARS coronaviruses in bat cells offers insights to their ancestral origins[4]	Not attempted	N/A
Coronavirus Infection and Diversity in Bats in the Australasian Region[5]	Not attempted	N/A
Detection of Coronavirus Genomes in Moluccan Naked-back Fruit Bats in Indonesia[6]	“African green monkey kidney (Vero E6), Yaeyama flying fox kidney (FBKT), and Leschenault’s rousette kidney (DemKT1)cells”	Not successful
A Bat-Derived Putative Cross-Family Recombinant	“Vero E6, BHK-21, MDCK, A549, HEp-2, CaCo-2 cells, as	Not successful

Coronavirus with a Reovirus Gene[7]	well as in an immortalized kidney cell line of Myotis Davidii”	
Bat Coronaviruses and Experimental Infection of Bats, the Philippines[8]	“Vero E6, Vero, Hrt18, A549, fcwf-4, BKT-1, Tb-1 Lu, or primary kidney cells derived from Leschenault rousette bats”	Not successful
Detection and full genome characterization of two beta CoV viruses related to Middle East respiratory syndrome from bats in Italy[9]	“VERO cells (African green monkey kidney), MARC-145 (foetal monkey kidney), HRT-18 (human colorectal adenocarcinoma), FRhK 4 (foetal rhesus kidney), LLC-Mk2 (rhesus monkey kidney) and TB1 LU (lung, Mexican free-tailed bat, “Tadarida brasiliensis mexicana”)”	Not successful
Longitudinal Surveillance of Betacoronaviruses in Fruit Bats in Yunnan Province, China During 2009–2016[10]	“Vero E6 and primary intestine cell lines of E. spelaea and R. leschenaulti”	Not documented

Elucidating the result of virus culture in [10]

Despite the best effort of trying to obtain the result for their attempted virus isolation in [10], no virus isolation results were documented anywhere within the article.

However, all the results on HKU9 and GCCDC1 were “amplified” from “selected positive samples”. All partial RdRp sequences were obtained by direct PCR amplification of RNA “extracted from bat fecal or anal samples”

And the “Tissue Tropism of batCoV HKU9 and GCCDC1-Related Virus” section used tissue samples directly obtained from the bats.

The only full-length genome recorded was “BatCoV HKU9-2202”, which was obtained from High Throughput sequencing “using an Illumina platform at Novogene (Beijing, China)” of a sample of “supernatant of homogenized intestine was centrifuged at 10,000×g for 10 min at 4 °C.” Indicating direct RNA isolation and sequencing from bat sample instead of cell culture samples.

No sequences indicated or deposited by the study were indicated to have come from cell culture. The “Electronic supplementary material” they provided were a list of primers they used for quantitative PCR.

We attempted a Keyword search of the article using keyword “isolated”, which returned 3 Citations on different articles. However, None of the articles indicated isolation of coronaviruses

related to HKU9 or GCCDC1, nor have experimentally isolated any Coronaviruses from their experiments. [11][12][13]

This indicate that this attempts at viral isolation also likely failed.

CONCLUSIONS

By using Keyword search and bioinformatic analysis of available material, we determined that all documented HKU9 strains and all GCCDC1 strains with a documented host, uses Bats of the Family Pteropodidae as it's natural host. In addition, there is significant evidence that the sequence without a documented host also came from Pteropodidae fruit bats. No HKU9 sequences have been documented to use Non-Pteropodidae Bats as it's host.

There is no evidence that HKU9 can use Rhinolophidae bats as hosts, at all.

With no documented success on isolating HKU9 in any cell lines tested, which included Human, Cat, Monkey and Bat cells, We conclude that there are no evidence that HKU9 is able to infect hosts other than the natural bat hosts they were detected or sequenced within.

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