**Title**

Full genome sequences of novel *Nobecoviruses* identified in endemic Madagascar fruit bats

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**Abstract**

Bats are natural reservoirs for both *Alpha*- and *Betacoronaviruses* and the hypothesized original hosts of five of seven known zoonotic coronaviruses. To date, the vast majority of bat coronavirus research has been concentrated in Asia, though coronaviruses are globally distributed; indeed, SARS-CoV and SARS-CoV-2-related *Betacoronaviruses* in the subgenus Sarbecovirus have been identified circulating in *Rhinolophid* bats in both Africa and Europe, despite the relative dearth of surveillance in these regions. In part with a long-term study examining the dynamics of potentially zoonotic viruses in three species of endemic Madagascar fruit bat (*Pteropus rufus, Eidolon dupreanum, Rousettus madagascariensis*), we carried out metagenomic Next Generation Sequencing on urine, throat, and fecal samples obtained from wild-caught individuals. We report detection of RNA derived from *Betacoronavirus* subgenus *Nobecovirus* in fecal samples from all three species and describe full genome sequences of novel *Nobecoviruses* in *P. rufus* and *R. madagascariensis.* Phylogenetic analysis indicates the existence of five distinct *Nobecovirus* clades, one of which is defined by the highly divergent sequence reported here from *P. rufus* bats*.* Madagascar *Nobecoviruses* derived from *P. rufus* and *R. madagascariensis* demonstrate, respectively, Asian and African phylogeographic origins, mirroring those of their fruit bat hosts. Bootscan recombination analysis indicates significant genomic reassortment has taken place in the spike, nucleocapsid, and NS7 accessory protein regions of the genome for viruses derived from both bat hosts. Madagascar offers a unique phylogeographic nexus of bats and viruses with both Asian and African phylogeographic origins, providing opportunities for unprecedented mixing of viral groups and, potentially, recombination. As fruit bats are handled and consumed widely across Madagascar for subsistence, understanding the landscape of potentially zoonotic coronavirus circulation is essential to mitigation of future zoonotic threats.

**Introduction**

In the past 20 years, bat-derived coronaviruses SARS-CoV, MERS-CoV, and SARS-CoV-2 have been responsible for two deadly epidemics and the ongoing COVID-19 pandemic (1–4). These coronaviruses (CoVs) are members of the *Betacoronavirus* genus, which, along with genus *Alphacoronavirus*, are primarily associated with bat hosts (1–4); the remaining CoV genera, *Gammacoronavirus and Deltacoronavirus,* are typically hosted by birds (5). The *Betacoronavirus* group can be further broken down into bat-associated subgenera *Sarbecovirus* (hosted by bats in family Rhinolophidae (6,7)), *Merbecovirus* (hosted by bats in family Vespertilionidae(8–10)), *Nobecovirus* (hosted by bats in family Pteropodidae (11–13)), and *Hibecovirus* (hosted by bats in family Hipposideridae (14,15). The final *Betacoronavirus* subgenus, *Embecovirus,* is primarily associated with rodent and bovid hosts (16,17). Since the emergence of SARS-CoV in 2002, there has been increasing interest in surveying potential hosts of coronaviruses and contributing new virus sequences to public databases, with most effort focused on sampling bats from Asia (18–25), the continent of origin for both the SARS-CoV epidemic and the SARS-CoV-2 pandemic. More recently, there has arisen a more concerted effort to survey the landscape of bat-borne coronaviruses in other regions, including Africa and Europe (11,13,26–30).

The family *Coronaviridae* is considered one of the most likely viral taxa to switch host species (31,32), in part because many CoVs utilize well-conserved cell surface receptors to present on a wide variety of mammalian host cells. The zoonotic Sarbecoviruses, SARS-CoV and SARS-CoV-2, for example, use the human cell surface receptor Angiotensin-converting enzyme 2 (ACE2) to gain entry into human cells (33,34), while many Merbecoviruses interact with the well-conserved vertebrate host cell receptor ﻿dipeptidyl peptidase 4 (DPP4) (35). Because CoVs are known to recombine with other CoVs, or more rarely, with other viral groups (26,38–41), there is concern that naturally-circulating CoVs presently unable to infect humans may acquire this ability in the future. This highlights the importance of widespread CoV surveillance aimed at characterizing the global landscape of CoV diversity potentially available for recombination. Several factors, which have been reviewed at length elsewhere (31,42,43), contribute to the CoV affinity for recombination, including the large CoV genome size supported by a unique proofreading mechanism in the RNA-dependent RNA polymerase (RdRp) (44–47), as well as a ‘copy choice’ template switching mechanism of RNA replication whereby RdRp physically detaches from one RNA template during replication and reattaches to an adjacent template, thus facilitating recombination in cases where multiple viruses may be coinfecting the same cell (48).

Madagascar is an island country in southeastern Sub-Saharan Africa, located in the Indian Ocean, ~400 km off the coast from Mozambique. Madagascar has been isolated from the African continent for over 170 million years and all surrounding landmasses for over 80 million years, allowing for the evolution of a unique and highly endemic floral and faunal assemblage (49). The country is home to 51 species of bat (50), some three-quarters of which are endemic and boast long evolutionary divergence times with sister species on both the African and Asian continents (51–53). A growing body of work has characterized the landscape of potentially zoonotic viruses in Madagascar bats, identifying evidence of circulating infection (through RNA detection or serology) with henipaviruses, filoviruses, lyssaviruses, and coronaviruses (29,54–56). Previously coronavirus surveillance efforts have identified *Alphacoronavirus* RNA in the Malagasy insectivorous bat, *Mormopterus jugalaris,* and *Betacoronavirus* RNA in all three endemic Malagasy fruit bat species, *Pteropus rufus, Eidolon dupreanum,* and *Rousettus madagascariensis* (62,100). Previous studies have demonstrated that this latter *Betacoronavirus* RNA clusters with subgenus *Nobecovirus* (29,55); *Nobecoviruses* have been previously described infecting *Pteropodidae* fruit bats across Asia and in both East and West Africa (21,28,57–60). Though Nobecoviruses are not known to be zoonotic, previous research has described widespread circulation of a recombinant Nobecovirus carrying an orthoreovirus insertion throughout Asia (21,60,61), highlighting the capacity for this viral subgenus to undertake rapid shifts in genomic organization which could lead to expanded host range. As both *Eidolon dupreanum* and *Rousettus madagascariensis* are known to co-roost with each other, and with several species of insectivorous bat (62), CoV recombination is a distinct concern in the Madagascar system. Though no *Rhinolophus* spp. bats, the typical host for ACE2-using *Sarbecoviruses*, inhabit Madagascar, the island is home to four species of Hipposiderid bat, which host the *Sarbecovirus-*adjacent and understudied *Hibecoviruses,* as well as several species of Vespertilionid bat, the most common hosts for the zoonotic *Merbecoviruses*.

Human-bat contact rates are high in Madagascar, where bats are consumed widely as a source of human food and frequently roost in close proximity to human settlements or natural tourist attractions (63–66). In addition to the natural CoV diversity circulating in Malagasy bats, several human coronaviruses are known to circulate widely in Madagascar, including the common cold-causing Embecoviruses, HCoV-OC43 and HCoV-HKU1, and, more recently, the zoonotic Sarbecovirus, SARS-CoV-2 (67–69). As spillback of SARS-CoV-2 into wildlife hosts and possible recombination with wildlife viruses remains a global concern (70), characterization of the genetic diversity of bat-borne coronaviruses in Madagascar and elsewhere in Africa is a critical public health priority. Here we contribute and characterize three full genome sequences of two novel *Nobecoviruses*, derived *R. madagascariensis* and *P. rufus* hosts. We define five distinct *Nobecovirus* clades in global circulation across Asia and Africa and assess these new *Nobecoviruses* for their past and future capacity for recombination.

**Materials and Methods**

*Bat Sampling*

As part of a longterm study characterizing the seasonal dynamics of potentially zoonotic viruses in wild Madagascar fruit bats, monthly captures of Malagasy pteropodid bats were carried out at species-specific roost sites in the Districts of Moramanga and Manjakandriana, Madagascar between 2018 and 2019 (*P. rufus:* Ambakoana roost, -18.513 S, 48.167 E; *E. dupreanum*: AngavoBe cave, -18.944 S, 47.949 E; AngavoKely cave = -18.933 S, 47.758 E; *R. madagascariensis*:Maromizaha Cave,-18.9623 S, 48.4525 E). In brief, bats were captured in nets hung in the tree canopy (*P. rufus*) or over cave mouths (*E. dupreanum, R. madagascariensis)* at dusk (17:00-22:00) and dawn (03:00-07:00), removed from nets, and processed under manual restraint following methods that have been previously described (54,71,72). Briefly, all animals were identified to species, sex, and age class (juvenile vs. adult), and fecal, throat, and urine swabs were taken from each individual, collected into viral transport medium, and frozen on site in liquid nitrogen. Post-sampling, swabs were transported to -80\*C freezers for longterm storage in the Virology Unit at Institut Pasteur of Madagascar.

This study was carried out ﻿in strict accordance with research permits obtained from the Madagascar Ministry of Forest and the Environment (permit numbers 019/18, 170/18, 007/19) and under guidelines posted by the American Veterinary Medical Association. All field protocols employed were pre-approved by the UC Berkeley Animal Care and Use Committee (ACUC Protocol # AUP-2017-10-10393), and every effort was made to minimize discomfort to animals.

*RNA Extraction*

RNA was extracted from a randomly selected subset of fecal (285), throat (143), and urine (196) swab samples in the Virology Unit at Institut Pasteur of Madagascar, with each sample corresponding to a unique individual from the field dataset. Samples undergoing mNGS corresponded to individuals captured in Feb-Apr, Jul-Sep and December 2018 or in January 2019. Water controls were extracted in conjunction with samples on each unique extraction day. Extractions were conducted using the Zymo Quick DNA/RNA Microprep Plus kit (ZYMO, Irvine, CA, USA), according to the manufacturer’s instructions and including the step for DNAse digestion. Post-extraction, RNA quality was checked on a nanodrop to ensure that all samples demonstrated 260/280 ratios exceeding 2 and revealed quantifiable concentrations. Resulting extractions were stored in freezers at -80\*C, then transported on dry ice to the Chan Zuckerberg Biohub (San Francisco, CA, USA) for library preparation and metagenomic Next Generation Sequencing (mNGS).

*Library Preparation and mNGS*

A subset of four randomly selected samples from each of three bat species was selected for additional quantification using an Invitrogen Qubit 3.0 Fluorometer and the Qubit RNA HS Assay Kit (ThermoFisher Scientific, Carlsbad, CA, USA). After quantification, all total RNA samples, along with water samples from Madagascar extractions, were manually arrayed into 96 well plates to enable automated high throughput mNGS library preparation. Based on the initial quantitation, a 2uL aliquot from each plated sample was diluted 1:9 on a Bravo liquid handling platform (Agilent, Santa Clara, CA, USA). A 5uL aliquot from each diluted sample was arrayed into a 384 well plate for input for the mNGS library prep. Samples derived from fecal, throat, and urine swab samples were arrayed on distinct 384 well plates for separate sequencing runs. An additional set of total RNA samples (a dilution series of total RNA isolated from cultured HeLa cells) and a set of local lab water samples were included on each 384 well plate as controls. The 384 well plate was transferred to a GeneVac EV-2 (SP Industries, Warminster, PA, USA) to evaporate the samples for miniaturized mNGS library preparation with the NEBNext Ultra II RNA Library Prep Kit (New England BioLabs, Beverly, MA, USA), per the manufacturer’s instructions, with the following modifications: a 25pg aliquot of External RNA Controls Consortium Spike-in mix (ERCCS, Thermo-Fisher) was added to each sample prior to RNA fragmentation; the input RNA mixture was fragmented for 8 min at 94°C prior to reverse transcription; and a total of 14 cycles of PCR with dual-indexed TruSeq adapters was applied to amplify the resulting individual libraries. An equivolume library pool was generated, and the quality and quantity of that pool was assessed via electrophoresis (High-Sensitivity DNA Kit and Agilent Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA), real-time quantitative polymerase chain reaction (qPCR) (KAPA Library Quantification Kit; Kapa Biosystems, Wilmington, MA, USA), and small scale sequencing (2 x146bp) on an iSeq platform (Illumina, San Diego, CA, US). Subsequent equimolar pooling of individual libraries and large-scale paired-end sequencing (2 × 146 bp) was performed on the Illumina NovaSeq sequencing system (Illumina, San Diego, CA, USA). The pipeline used to separate the sequencing output into 146-base-pair pair-end read FASTQ files by library and to load files onto an Amazon Web Service (AWS) S3 bucket is available on GitHub at <https://github.com/czbiohub/utilities>.

*IDSeq*

Raw reads from Illumina sequencing were host-filtered, quality-filtered, and assembled on the IDseq (v3.10, NR/NT 2019-12-01) platform, a cloud-based, open-source bioinformatics platform designed for microbe detection from metagenomic data (73), using a host background model of “bat” compiled from all publicly available full-length bat genomes in GenBank at the time of sequencing. Samples were deemed “positive” for coronavirus infection if IDseq successfully assembled at least two nucleotide or protein-BLAST derived contigs with an average read depth >2 reads/nucleotide, mapping to any CoV reference accession number. To clarify that no positives were missed from IDseq, all non-host contigs assembled in IDseq underwent offline BLASTn and BLASTx (74) against a reference database constructed from all full-length reference sequences and protein reference sequences for *Alpha-* and *Betacoronavirus* available in NCBI Virus. Step-by-step instructions for our offline BLAST protocol can be accessed in our publically available GitHub repository at: https://github.com/brooklabteam/Mada-Bat-CoV/.

*Genome Annotation and BLAST*

Three full genome-length Nobecovirus contigs returned from IDseq (two from *R. madagascariensis* and one from *P. rufus*) were aligned with Nobecovirus homologs from NCBI (see ‘Phylogenetic Analysis’) and annotated in the program Geneious Prime (2020.0.5). We then used NCBI BLAST and BLASTx to query identity of our full length recovered genomes and their respective translated proteins to publicly available sequences in NCBI (74). We queried identity to reference sequences for four previously described *Nobecovirus* strains (accession numbers: MG762674 (HKU9), NC\_030886 (GCCDC1), MK211379 (GX2018), and NC\_048212 (*Eidolon helvum* sequences), as well as to the top BLAST hit overall. In instances where a putative gene recovered no hits to homologs via BLASTx, we instead queried the HHPred interactive server for protein and homology detection (75).

*Phylogenetic Analysis*

Contigs returned from IDseq were next combined with publicly available coronavirus sequences in NCBI to perform phylogenetic analysis. We carried out three major phylogenetic analyses, building (a) a full-genome *Betacoronvirus* phylogeny, (b) a *Betacoronavirus* RdRp phylogeny corresponding to a conserved 259 bp fragment of the RNA-dependent RNA polymerase gene encapsulated in the CoV Orf1b, and (c) four amino acid phylogenies derived from translated nucleotides corresponding to the spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins of a subset of full length genomes. Detailed methods for the construction of each phylogeny are available at <https://github.com/brooklabteam/Mada-Bat-CoV/>.

Briefly, our full genome phylogeny was comprised of 122 unique NCBI records, corresponding to all available full genome sequences with bat hosts under NCBI taxon ids, *Betacoronavirus* (694002), unclassified *Betacoronavirus* (696098), *Betacoronavirus* sp. (1928434), unclassified Coronaviridae (1986197), or unclassified Coronavirinae (2664420) (107 records), in addition to all full genome *Betacoronavirus* (694002)**reference** sequences with a non-bat host (14 records), plus one *Gammacoronavirus* outgroup (accession number NC\_010800). The full genome phylogeny additionally included three full length Madagascar Nobecovirus sequences returned from IDseq (two from *R. madagascariensis* and one from *P. rufus*), which are described in this paper for the first time.

Our *Betacoronavirus* RdRp phylogeny consisted of an overlapping subset of a 259 bp RdRp fragment derived from *Betacoronviruses* previously described in Madagascar fruit bats (55) (7 records), in addition to the same RdRp fragment extracted from 17 near-full length *Nobecovirus* sequences, two RdRp *Nobecovirus* fragments, and 17 full length reference sequences for other *Betacoronavirus* subgenera available in NCBI Virus. This phylogeny also included seven Madagascar Nobecovirus sequences encompassing the RdRp fragment of interest, which were returned from the assembly in IDseq (four from *R. madagascariensis,* two from *P. rufus,* and one from *E. dupreanum*), in addition to the RdRp fragment of our *Gammacoronviru*s outgroup.

Lastly, our amino acid phylogenies consisted of S, E, M, and N gene extractions from the same representative set of near-full genome length sequences used in the RdRp analysis: the same 17 full-length *Betacoronavirus* reference sequences, 17 near full-length *Nobecovirus* sequences, and the one *Gammacoronavirus* outgroup, in addition to our three full genome Madagascar sequences derived from *R. madagascariensis* and *P. rufus*. Gene extractions were carried out using annotation tracks reported with each accession number in NCBI or, in cases where annotations were unavailable, genes were manually annotated and extracted in Geneious Prime based on alignment to homologs. After nucleotide extraction, genes were translated prior to alignment.

After compiling sequences for each disparate phylogenetic analysis, sequence subsets for the full-length, RdRp, and four amino acid phylogenies were aligned in MAFFT v.7 (76,77) using default parameter values. Alignments were checked manually for quality in Geneious Prime, and the RdRp aligment was trimmed to a 259 bp fragment conserved across all sequences in the subset. All sequence subsets and alignment files are available for public access in our Github repository: <https://github.com/brooklabteam/Mada-Bat-CoV/>.

After quality control, alignments were sent to Modeltest-NG (78) to assess the best fit nucleotide or amino acid substitution model appropriate for the data, then to RAxML-NG (79) to construct the corresponding maximum likelihood (ML) tree. Following best practices outlined in the RAxML-NG manual, twenty ML inferences were made on each original alignment and bootstrap replicate trees were inferred using Felsenstein’s method (80), with the MRE-based bootstopping test applied after every 50 replicates (81). Bootstrapping was terminated once diagnostic statistics dropped below the threshold value and support values were drawn on the best-scoring tree. Resulting phylogenies were visualized in R v.4.0.3 for MacIntosh, using the package ‘ggtree’ (82).

*Recombination Analysis*

Full length *Nobecovirus* sequences derived from IDseq were analyzed for any signature of past recombination. All three full length Madagascar *Nobecovirus* genomes were aligned with full genome sequences corresponding to two disparate *Nobecovirus* lineages: the HKU9 lineage (EF065514-EF065516, HM211098-HM211100, MG693170, NC\_009021, MG762674) and the *Eidolon helvum* Africa lineage (MG693169, MG693171-MG693172, NC\_048212). Alignment was conducted in MAFFT v.7 (76,77) using default parameter values. *Nobecovirus* sequences corresponding to the GCCDC1 (21,60) and GX2018/BatCoV92 (103,132) genotypes were omitted from recombination analyses because inserted genes and/or genetic material upstream from the nucleocapsid in the corresponding genomes interfered with the alignment.

After alignment, genomes were analyzed for recombination in the program SimPlot (v.3.5.1). Similarity plots, which compute identity across all genomic positions in an alignment, were generated using the *P. rufus* and, subsequently, the *R. madagascariensis* genomes as query sequences, the HKU9 and *Eidolon helvum* African *Nobecovirus* clades as references, and the corresponding Madagascar sequence as the alternative. Bootscan analyses were conducted on the same alignment, using the same query and reference inputs. Both Similarity and Bootscan analyses were carried out using a window size of 200bp and a step size of 20bp.

*Nucleotide Sequence Accession Numbers*

All three annotated full-length genome sequences (two from *R. madagascariensis,* one from *P. rufus*), plus four additional RdRp gene fragment sequences (two from *R. madagascariensis,* one from *P. rufus*, and one from *E. dupreanum*) were submitted to NCBI and assigned accession numbers XXXXX-XXXX (pending).

**Results**

*Prevalence of CoV Sequence Detection in Field Samples*

IDseq assembly generated at least two nucleotide or protein-BLAST derived contigs with an average read depth > 2 reads/nucleotide, mapping to any CoV reference accession number, in 28/285 (9.82%) of fecal samples and in 2/196 (1.00%) of urine samples. Given low prevalence, the latter urine positives likely signified field contamination with fecal excrement upon urine swab collection, as bats often excrete both substances simultaneously under manual restraint. None of the 143 throat swabs assayed demonstrated evidence of CoV infection.

Prevalence in feces varied slightly across species, with 4/44 (9.09%) of *P. rufus* samples*,* 16/145 (11.03%) of *E. dupreanum* samples, and 8/96 (8.33%) of *R. madagascariensis* samples testing positive for CoV infection. Juveniles demonstrated higher CoV prevalence than adults for *P. rufus* and *E. dupreanum* but not for *R. madagascariensis*. Juvenile vs. adult prevalence was 3/15 (20%) vs. 1/29 (3.45%) for *P. rufus,*  5/13 (38.46%) vs. 11/132 (8.33%) for *E. dupreanum*, and 0/13 (0%) vs. 8/83 (9.64%) for *R. madagascaiensis* (**Figure 1A**)*.* Prevalence varied seasonally across all three species, peaking coincidentally in adult and juvenile populations for *P. rufus* and *E. dupreanum* and all species excepting *R. madagascariensis,* with the highest prevalences observed during the wet season months of February-April when late stage juveniles are present in the population, following each species’ birth pulse (**Figure 1B**).

*Genome Annotation and BLAST*

Three full genome or near-full genome length contigs were recovered from IDseq for *Nobecoviruses* derived from *R. madagascariensis* (two genomes: 28,980 and 28,926 bps in length) and *P. rufus* (one genome: 29,122 bps in length). In all three genomes, we successfully annotated ORF1ab (including RdRp) and structural proteins S (spike), E (envelope), M (matrix), and N (nucleocapsid), in addition to accessory genes NS3, NS7a, and NS7b (**Figure 2**). Genomes derived from *R. madagascariensis* appeared slightly more complex than those derived from *P. rufus,* allowing for annotation of one additional accessory gene, NS7c, which has been described previously in recombinant *Nobecovirus* sequences of the GCCDC1 lineage (37,105).

In addition to major proteins, we successfully identified several 6 bp motifs corresponding to the 5’-ACGAAC-3’ core Transcription Regulatory Sequence (TRS) common to many *Betacoronaviruses,* including SARS-CoV and previously described in *Nobecoviruses* of the GCCDC1 and GX2018/BatCoV92 lineages (132–134). For most genes, these TRS elements were located a short distance upstream from the corresponding gene (**Table 1**). Elements identified in the two *R. madagascariensis* genomes were largely comparable, suggesting that these two sequences could represent slight variations in the same virus lineage—though the intergenic region for one putative *R. madagascariensis* TRS located upstream from the N gene was substantially longer than the other, thus highlighting the dynamic nature of the CoV genome in this region. This region corresponds to the site of the previously described orthoreovirus gene insertion in *Nobecovirus* genotype GCCDC1 (133). Some putative TRS elements showed variation from the 5’-ACGAAC-3’ core motif (including some which recapitulated the 5’-AAGAA-3’ motif common to SARS-CoV-2 (135)). TRS variations may be indicative of variation in gene expression across individual bats and/or species.

BLAST analysis of the full genome indicated that the *P.rufus* *Nobecovirus* sequence is highly divergent, demonstrating 72-73% identity to all previously described *Nobecovirus* clades, with the top blast association to HKU9 (**Table S1**). By contrast, *Nobecovirus* genomes derived from *R. madagascariensis* demonstrated high identity (~95%) to *Eidolon helvum Nobecovirus* lineages circulating in Africa. BLASTx analysis of individual genes from viruses derived from both Madagascar species demonstrated the highest identity with previously described *Nobebecovirus* sequences in the Orf1b region (which includes RdRp) for both *P. rufus* and *R. madagascariensis* viruses (70.08% identity for *P. rufus* to HKU9and 99% identity for *R. madagascariensis* to *E. helvum*). By contrast, both *P. rufus* and *R. madagascariensis* genomes demonstrated substantial divergence from all known homologs in the S and NS7 regions of their genomes, showing only 45.59-63.64% identity to the closest hits across these regions. The *P. rufus Nobecovirus* was similarly divergent in the N gene, though *R. madagascariensis Nobecoviruses* demonstrated high (86-87%) identity to *E. helvum* genotypes in this region.

*Phylogenetic Analysis*

Phylogenetic analysis of full length *Betacoronavirus* genomes confirmed that both *P. rufus* and *R. madagascariensis* genomes cluster in the *Nobecovirus* subgenus of the *Betacoronaviruses*, with the divergent *P. rufus* forming its own distinct clade and both *R. madagascariensis* genomes grouping with the previously described *Eidolon helvum* reference sequence from Cameroon (102) (**Figure 3A**). We observed distinct groupings of five main *Nobecovirus* lineages in our phylogeny: (a) the largely Asian-derived HKU9 sequences, (b) the African *E. helvum-*derivedsequences (now including new *R. madagascariensis* genomes), (c) the orthereovirus recombinant GCCDC1 genomes, (d) the GX2018/BatCoV92 genomes described from China and Singapore, and (e) the divergent *P. rufus* genome contributed here from Madagascar. Intriguingly, the *P. rufus* genome groups ancestral to all other *Nobecoviruses,* followed by the *E. helvum/R. madagascariensis* African lineage, with the Asian genotypes forming three distinct (and more recent) clades corresponding to genotypes HKU9, GCCDC1, and Gx2018/BatCoV92.Further phylogenetic analysis of a 259bp fragment of the RdRp gene reconfirmed these groupings and suggested the presence of at least two distinct genetic variants within the *P. rufus* lineage (**Figure 3B**). One RdRp fragment derived from feces of the third Malagasy fruit bat, *Eidolon dupreanum,* grouped within the *E. helvum/R. madagascariensis* African *Nobecovirus* lineage*,* consistent with previous reporting (100). Characterization of the full length genome of this virus will be needed to clarify whether it represents a genetic variant of or a distinct genotype from the *R. madagascariensis* virus. Phylogenetic analysis of the RdRp fragment allowed for inclusion of one partial *Nobecovirus* sequence derived from *E. helvum* bats in Kenya (HQ728482), which also grouped within the *E. helvum/R. madagascariensis* African clade, confirming the distribution of this genotype across West and East Africa and into the Indian Ocean*.* Notably, one partial Cameroonian *E. helvum* sequence (MG693170) clustered with HKU9 sequences from Asia, rather than within the *E. helvum/R. madagascariensis* African clade. These findings suggest that both “African” and “Asian” *Nobecovirus* lineages are likely broadly geographically distributed.

Amino acid phylogenies computed from translated protein alignments of the S, E, M, and N *Betacoronavirus* structural genes (**Figure 4 A-D**, respectively) further confirmed evolutionary relationships suggested in Figure 3. S, M, and N gene phylogenies demonstrated distinct groupings of five main *Nobecovirus* lineages outlined above, while in the E gene phylogeny, the *P. rufus* sequence grouped adjacent to the single Cameroonian-derived *E. helvum* sequence within the HKU9 clade.

*Recombination Analysis*

SimPlot analysis confirmed the evolutionary distinctiveness of the *P. rufus Nobecovirus* genome, which showed <50% identity to HKU9, *E. helvum,* and *R. madagascariensis* genotypes across the majority of its genome length (**Figure 5A**). Consistent with BLAST results, the *P. rufus Nobecovirus* genome demonstrated the highest similarity to previously described sequences in the Orf1b region, which includes RdRp. The *R. madagascariensis Nobecoviruses*, by contrast, showed >90% similarity to the *E. helvum* African lineage throughout Orf1ab, but both *P. rufus* and *R. madagascariensis* sequences diverged from all other reference genomes in the first half of the spike protein, which corresponds to the S1 subunit and includes the receptor binding domain that mediates viral entry into host cells (136). Further divergence for both *P. rufus* and *R. madagascariensis Nobecoviruses* was observed in the N structural protein and in the NS7 accessory genes. Bootscan analysis further confirmed these findings, showing that the *P. rufus Nobecovirus* clusters with HKU9 lineages across Orf1ab, NS3, E, and M genes but demonstrates evidence of recombination with *E. helvum/R. madagascariensis* African lineages in the S (particularly S1), N, and NS7 genes (**Figure 5B**). Similarly, bootscanning demonstrated that *R. madagascariensis Nobecoviruses* group with the *E. helvum* lineage across Orf1ab, NS3, E, and M but show evidence of recombination with HKU9 and *P. rufus Nobecovirus* in S (again, particularly S1), N, and NS7 genes (**Figure 5B**), thus highlighting the dynamic nature of these regions of the Nobecovirus genome.

**Discussion**

We here contribute three full-length genome sequences and several RdRp fragments to public NCBI repositories; these sequences correspond to at least two novel *Nobecoviruses* derived from wild Madagascar fruit bats, *Pteropus rufus* and *Rousettus madagascariensis,* with evidence of additional genetic variants circulating in *Eidolon dupreanum,* as well. Phylogenetic analyses suggest that previously-described *Nobecoviruses* can be grouped in five general clades: (a) the HKU9 lineage of largely Asian origins, (b) the mostly African-distributed lineage derived from *E. helvum* bats (which contains our *R. madagascariensis* and *E. dupreanum* sequence contributions), (c) the recombinant orthoreovirus lineage, termed GCCDC1, which has been previously reported from China and Singapore, (d) the genetically variant GX2018/BatCoV92 lineage, also known from China and Singapore, and (e) a novel, divergent clade corresponding to the newly-described *P. rufus* genome. Importantly, though largely characterized in Asia, HKU9 *Nobecovirus* genotypes have been identified in West Africa (Cameroon), and *Eidolon helvum* lineages have been characterized across West (Cameroon) and East (Kenya) Africa, as well as in the central Indian Ocean (Madagascar). These findings suggest that different *Nobecovirus* clades may be more broadly geographically distributed than has been previously recognized. To our knowledge, no *Nobecoviruses* have been identified from pteropodid fruit bats in Australia; characterization of any CoVs infecting these bats, which are known to host important, zoonotic henipaviruses and lyssaviruses, would do much to enhance our understanding of the phylogeography of the *Nobecovirus* clade. Madagascar represents a unique phylogeographic melting pot, with flora and fauna—and corresponding viruses—of both African and Asian descent, offering opportunities for mixing of largely disparate viral groups. This mixing is important in light of the CoV penchant for recombination, which can allow viruses from one clade to gain function through acquisition of genetic material from another, thus facilitating rapid changes in host range (26,38–41).

*Nobecoviruses* are not known to be zoonotic and have been thus far described exclusively infecting fruit bats hosts of the Old World bat family, Pteropodidae. Nonetheless, the *Nobecovirus* subgenus demonstrates a CoV-characteristic tendency to recombine, as evidenced by circulation of the widespread GCCDC1 lineage in Asia, which carries a p10 gene insertion between the N structural protein and NS7a accessory protein towards the 3’ end of the genome. This GCCDC1 orthoreovirus insertion was not detected among viruses in our dataset, though, anecdotally, mNGS of fecal, throat, and urine samples collected in our sampling did identify evidence of orthreovirus infection in several throat swabs derived from *E. dupreanum* bats*,* highlighting the potential for recombination opportunities between these two viral groups in the future*.* Importantly, we observed the presence of some variable genetic material downstream from the N gene and upstream from the NS7a gene in the divergent *P. rufus Nobecovirus* genome; *Nobecoviruses* clustering in the GX2019/BatCoV92 lineage also carry a unique coding sequence in this region, highlighting the dynamic nature of the 3’ end of the CoV genome. Notably, recombination analyses suggested substantial genomic rearrangement has taken place in this region of both *R. madagascariensis* and *P. rufus-*derived *Nobecoviruses.* Recombination and/or mutation at the 3’ end of the CoV genome may modulate viral replication ability, since several regulatory sequences and accessory genes (e.g. NS7) are defined in this region. Viral replication ability may be further impacted by variation in TRS motifs, which regulate expression of corresponding genes. We identified putative TRS sequences corresponding to all structural and non-structural genes identified in all three contributed *Nobecovirus* genomes; while the majority of these TRS motifs recapitulated the well-conserved 5’-ACGAAC-3’ *Betacoronavirus* core sequence (132–134), variation in a subset of genes across species and individuals (e.g. differing motifs between two *R. madagascariensis*-derived gneomes) may correspond to variation in gene expression.

Recombination potential is a particular cause for concern in cases where viruses that lack the ability to infect human cells may acquire this zoonotic capacity through genetic exchange with other viruses coinfecting the same host. Indeed, the original SARS-CoV is believed to have acquired its capacity to bind human ACE2 through a recombination event with ACE2-using *Sarbecoviruses* in the disparate SARS-CoV-2 clade. *Sarbecoviruses,* in particular, are known to recombine frequently, giving rise to new genetic variants, in regions where different species of Rhinolophid bat hosts co-roost and share viruses. Cave-resident Malagasy fruit bats, *E. dupreanum* and *R. madagascariensis,* are known to co-roost with each other and with several species of insectivorous bat (62), which could facilitate *Nobecovirus* recombination. The observed similarity in *Nobecovirus* sequences derived from *E. dupreanum* and *R. madagascariensis* (which cluster in the same lineage), as compared with disparate sequences derived from tree-roosting *P. rufus,* suggest that some CoV genetic exchange may have already taken place between bats with overlapping habitats. To date, zoonotic potential has not been demonstrated for any previously described *Nobecoviruses,* and Rhinolophid bats associated with ACE2 usage are not resident in Madagascar. Nonetheless, bats in family Vespertilionidae, the family most commonly associated with zoonotic *Merbecoviruses* (8–10), are widespread in Madagascar, and *Mormopterus jugularis,* a known Molossidae bat host for *Alphacoronaviruses* of undetermined zoonotic potential, has been described co-roosting with *R. madagascariensis.* Bootscan analyses identified signatures of recombination in the S1 subunit of both *P. rufus* and *R. madagascariensis Nobecovirus* spike proteins, suggesting that this region of the genome, which modulates host range through cell surface receptor binding, may be under selective pressure.

In addition to posing risk for future zoonoses, *Nobecoviruses* derived from wild, Madagascar fruit bats could provide unprecedented genetic material for recombination to existing human coronaviruses already in circulation across the island—most notably SARS-CoV-2. At the time of this writing, COVID-19 infections remain widespread and vaccination limited across Madagascar. Previous work has assessed the risk of reverse zoonosis, or ‘spillback’ of SARS-CoV-2 from human to bat populations in the United States, concluding that high human caseloads and frequent human-bat contact rates in research settings pose both conservation risks to naïve bat populations presented with a novel pathogen, as well as human health risks presented by the possible establishment of secondary wildlife reservoirs for SARS-CoV-2 capable of sourcing future epidemics or the generation of unique viral variants through human-wildlife virus recombination. Bat-human contact rates are higher, on average, in Madagascar than in the US, as bats are consumed across the island for subsistence and frequently found roosting in human establishments or human-adjacent habitats (63–66). SARS-CoV-2 has already demonstrated its capacity for successful reserve zoonosis and adaptation to non-human hosts, in the case of farmer-sourced infections of mink in Finland, underscoring the legitimacy of these concerns.

Prevalence of coronavirus RNA by sequence detection in fecal samples averaged around 10% across all three Malagasy fruit bat species examined in our study, consistent with CoV prevalences reported in wild bat species elsewhere (73,74). One previous study of CoV circulation in Madagascar fruit bats reported much lower prevalence of infection in *E. dupreanum* and *R. madagascariensis*-derived fecal samples*,* respectively 1/88 (1.14%) and 0/141 (0%), as compared with a 13/88 (14.77%) prevalence in *P. rufus-*derived feces. As in our study, this previous work found no positive infections in throat swab samples, supporting a predominant gastrointestinal tropism for CoVs in this fruit bat system, in contrast to the respiratory infections more commonly observed in humans. Another study in the West Indian Ocean provided more information about CoV prevalences of xx() in xx, xx() in xx, and XX() in xx in Madagascar (around 5%) (28). Consistent with previous findings (XXX), we observed the highest prevalence of CoV infection in *P. rufus* and *E. dupreanum* juveniles. We hypothesize that the absence of juvenile infection identified in *R. madagascariensis* bats in our study could be due to the staggered nature of the birth pulse for these three species: Madagascar fruit bats birth in three successive birth pulse waves, led by *P. rufus* in October, and followed by *E. dupreanum* in November and *R. madagascariensis* in January and February. It is possible that the bulk of juvenile *R. madagascariensis* bats sampled in the month February were yet too young to be infected with CoVs (perhaps still protected via inherited maternal immunity) and that by the time of secondary sampling in April, juveniles were large enough to be erroneously classed as adults (size range variation is more limited in the smaller *R. madagascariensis* than in the two other Malagasy fruit bat species).

Our work emphasizes the importance of longitudinal ecological studies in identifying viral shedding events in transiently-infected wildlife hosts across multiple age and reproductive classes, while also highlighting the enhanced evolutionary and functional virological inference captured in a full genome sequence. More thorough studies documenting the seasonal dynamics of bat-borne CoVs, which elucidate genetic variation within and between species that share habitats will be essential to understanding CoV recombination, host shifting, and zoonotic potential. Replication of such studies across the global range of both coronaviruses and their bat hosts in needed to assess the landscape of future zoonotic risks and present opportunities for intervention and mitigation.

**Conflict of Interest:**

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

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**Figure Legends**

**Figure 1.** **(A)** Map of sampling sites for *P. rufus*, *E. dupreanum*, and *R. madagascariensis* in the Districts of Moramanga and Manjakandriana, Madagascar. Pie charts correspond to coronavirus prevalence in juveniles vs. adults across all three species: 3/15 (20%) vs. 1/29 (3.45%) for *P. rufus,*  5/13 (38.46%) vs. 11/132 (8.33%) for *E. dupreanum*, and 0/13 (0%) vs. 8/83 (9.64%) for *R. madagascaiensis*. Pie circle size corresponds to sample size on a log-10 scale. **(B)** Seasonal variation in adult vs. juvenile CoV prevalence by species, from sites depicted in (A).

**Figure 2.** Genome structure of novel *Nobecoviruses* derived from *P. rufus* and *R. madagascariensis* fruit bats. TRS locations are highlighted by red arrows, and genes are distinguished by color, with orange corresponding to ORF1a and ORF1b, and various shades of blue to structural proteins S, E, M, and N. Accessory genes NS3, NS7a, NS7b, and NS7c (*R. madagascariensis* genomes only) are depicted in powder blue.

**Figure 3. (A)** Maximum Likelihood phylogeny of full genome *Betacoronavirus* sequences, (RAxML-NG, GTR+I+G4) and **(B)** RdRp phylogeny of a 259bp fragment of *Betacoronavirus* Orf1b (RAxML-NG, TVM+I+G4). Bootstrap support values computed using Felsenstein’s method (80) are visualized on tree branches. In both (A) and (B), novel Madagascar sequences are highlighted in yellow, and tip points are colored by *Betacoronavirus* subgenus, corresponding to the legend. Tip shape indicates whether the virus is derived from a bat (triangle) or non-bat (circle) host. Both trees are rooted in turkey *Gammacoronavirus,* accession number NC\_010800.

**Figure 4.** Maximum Likelihood amino acid phylogenies corresponding to translated sequences of the **(A)** spike, **(B)** envelope, **(C)** matrix, and **(D)** nucleocapsid *Betacoronavirus* proteins. All phylogenies were computed in RAxML-NG, using respective amino acid substitution models (A) WAG+I+G4+F, (B) LG+G4, (C) LG+I+G4, and (D) LG+I+G4+F. Bootstrap support values computed using Felsenstein’s method (80) are visualized on tree branches. In (A-D) novel Madagascar sequences are highlighted in yellow, and tip points are colored by *Betacoronavirus* subgenus, corresponding to the legend. Tip shape indicates whether the virus is derived from a bat (triangle) or non-bat (circle) host. Both trees are rooted in turkey *Gammacoronavirus,* accession number NC\_010800.

**Figure 5. (A)** Similarity and **(B)** Bootscan plots computed in SimPlot (v.3.5.1), using a query sequence of *P. rufus* (top panels) and *R. madagascariensis* (bottom panels)*-*derived *Nobecovirus* sequences, with reference sequences corresponding to HKU9 (EF065514-EF065516, HM211098-HM211100, MG693170, NC\_009021, MG762674) and *Eidolon helvum* Africa-derived (MG693169, MG693171-MG693172, NC\_048212 ) *Nobecovirus* lineages. Line color indicates similary (A) and bootscan grouping (B) of the query sequence with the corresponding *Nobecovirus* genotype, along disparate regions of the CoV genome, as indicated by the colored bar at the bottom of each plot. All analyses were carried out using a window size of 200bp and a step size of 20bp.

**Table 1. Putative Transcription Regulatory Sequences in novel *Nobecoviruses* from Madagascar fruit bats.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Coronavirus** | **ORF** | **TRS location (nt)** | **Leader TRS (nt)** | **TRS region** | **Intergenic TRS** | **Distance from TRS to AUG (nt)** |
| P\_rufus\_AMB140 | ORF1ab | 65-70 | UGAA | ACGAAC | UUAAU | 21 |
|  | S | 20667-20672 | GUGA | ACGAAC | UUGUC | 69 |
|  | NS3 | 24593-24598 | AAAG | ACGAGC | UUA**AUG** | 3 |
|  | E | 25240-25245 | UUUA | ACGAAC | GUC**AUG** | 3 |
|  | M | 25451-25456 | UUGA | ACGAAC | AACAA | 14 |
|  | N | 26170-26175 | UUGA | ACGAAA | UUAAA | 6 |
|  | NS7a | 27651-27656 | AAGC | AGCAAC | AGAAA | 70 |
|  | NS7b | 28441-28446 | UUGA | ACGAAG | **AUG** | 0 |
| R\_madagascariensis\_MIZ178 | ORF1ab | 55-60 | GUUG | AAGAAC | UUUAA | 7 |
|  | S | 20790-20795 | UUGA | ACGAAC | UAAAA | 13 |
|  | NS3 | 24575-24580 | UUGA | ACGAAC | UUGUU | 20 |
|  | E | 25293-25298 | GUAA | ACGAAC | UGUAU**AUG** | 5 |
|  | M | 25508-25513 | GAUG | UCGAAC | UAUA**AUG** | 4 |
|  | N | 26255-26260 | UUGA | ACGAAC | AACAA | 17 |
|  | NS7a | 27674-27679 | UUGA | ACGAAC | CAAUU**AUG** | 5 |
|  | NS7b | 28046-28051 | UUGA | ACGAAC | **AUG** | 0 |
|  | NS7c | 28256-28261 | UUUU | ATCAAC | CCGGG | 27 |
| R\_madagascariensis\_MIZ240 | ORF1ab | 119-124 | UUGA | ACGAAC | CU**AUG** | 2 |
|  | S | 20854-20859 | UUGA | ACGAAC | UAAAA | 13 |
|  | NS3 | 24666-24671 | UUGA | ACGAAC | UUGUU | 20 |
|  | E | 25384-25389 | GUAA | ACGAAC | UGUAUA | 6 |
|  | M | 25599-25604 | GAUG | UCGAAC | UAUAA | 8 |
|  | N | 26346-26351 | UUGA | ACGAAC | AACAA | 154 |
|  | NS7a | 27771-27776 | UUGA | ACGAAC | CAAUU**AUG** | 5 |
|  | NS7b | 28216-28221 | UUGA | ACGAAC | **AUG** | 0 |
|  | NS7c | 28353-28358 | GUCG | AGAAAG | AGACC | 59 |

**Table S1. BLAST identity of novel Madagascar *Nobecoviruses* to publicly available reference genomes**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Virus** | **Nucleotide or amino acid** | **β-HKU9**  **(MG762674)** | **β--GCCDC1**  **(NC\_030886)** | **β-BtRt-BetaCoV/GX2018**  **(MK211379)** | **β-Eidolon\_helvum/Cameroon/2013**  **(NC\_048212)** | **Top BLAST hit** | **Accession number of top BLAST hit** |
| P\_rufus\_AMB130 | Genome\* | 73.15\* | 72.87\* | 73.28\* | 73.54\* | Bat coronavirus HKU9, complete genome | EF065514 |
|  | ORF1a | 47.67 | 53.7 | 53.77 | 48.25 | ORF1a [Bat coronavirus] | AWV67046 |
|  | ORF1b | 70.08 | 75.2 | 74.68 | 76.10 | ORF1ab polyprotein [Bat coronavirus] | YP\_009824989 |
|  | S | 45.59 | 47.3 | 46.7 | 46.47 | spike glycoprotein [Bat coronavirus] | QEH60463 |
|  | NS3 | 40.38 | 41.18 | 4.6† | 41.67 | hypothetical protein [Bat coronavirus HKU9] | ADM33567 |
|  | E | 50.68 | 47.95 | 50.63 | 46.58 | envelope protein [Eidolon bat coronavirus/Kenya/KY24/2006 | ADX59468 |
|  | M | 58.56 | 59.73 | 60.55 | 62.44 | Membrane glycoprotein [Bat coronavirus HKU9] | ABN10930 |
|  | N | 50 | 50.61 | 50.84 | 48.27 | N protein [Rousettus bat coronavirus HKU9] | AVP25400 |
|  | NS7a† | 3.3† | 2.1† | 1.9† | 2.2† | CMRF35-like molecule 1 viral protein [Murine norovirus GV/CR10/2005/USA]† | 5OR7† |
|  | NS7b | 40.28 | 0.1† | 63.64 | 40 | NS7b [Rousettus bat coronavirus HKU9] | AVP25412 |
| R\_madagascariensis\_MIZ178 | Genome\* | 77\* | 75.08\* | 75.88\* | 95.15\* | Bat coronavirus isolate CMR900 | MG693169 |
|  | ORF1a | 60.59 | 59.36 | 59.84 | 96.03 | ORF1a [Bat coronavirus] | AWV67062 |
|  | ORF1b | 82.02 | 81.09 | 82.54 | 99.02 | ORF1ab polyprotein [Bat coronavirus] | YP\_009824989 |
|  | S | 49.84 | 50.86 | 51.23 | 66.61 | Spike protein [Bat coronavirus] | AWV67064 |
|  | NS3 | 46.93 | 44.44 | 39.02 | 89.32 | ORF3 protein [Eidolon bat coronavirus Kenya/KY24/2006] | ADX59467 |
|  | E | 58.11 | 55.41 | 64.86 | 92 | envelope protein [Eidolon bat coronavirus/Kenya/KY24/2006] | ADX59468 |
|  | M | 70.70 | 70.75 | 71.09 | 91.98 | membrane protein [Bat coronavirus] | YP\_009824992 |
|  | N | 61.19 | 59.25 | 63.01 | 86.6 | capsid [Bat coronavirus] | AWV67051 |
|  | NS7a | 55.65 | 25.89 | 1.5† | 8.7† | hypothetical protein ORFx [Bat coronavirus] | AWV67068 |
|  | NS7b | 0.1† | 1.3† | 46.67 | 0.8† | hypothetical protein [Bat coronavirus HKU9] | ADM33571 |
|  | NS7c | None | 28.57 | 0† | 45.89 | hypothetical protein ORFy [Eidolon bat coronavirus/Kenya/KY24/2006] | ADX59472 |
| R\_madagascariensis\_MIZ240 | Genome\* | 77.06 | 75.25 | 75.99 | 95.31 | Bat coronavirus isolate CMR891-892 | MG693171 |
|  | ORF1a | 60.54 | 59.41 | 59.77 | 96.15 | ORF1a [Bat coronavirus] | AWV67062 |
|  | ORF1b | 81.91 | 81.12 | 82.28 | 99.13 | ORF1ab polyprotein [Bat coronavirus] | YP\_009824989 |
|  | S | 51.05 | 50.6 | 51.37 | 66.61 | Spike protein [Bat coronavirus] | AWV67064 |
|  | NS3 | 45.81 | 44.44 | 4.7† | 89.74 | ORF3 [Bat coronavirus] | AWV67065 |
|  | E | 58.11 | 55.41 | 64.86 | 92 | envelope protein [Eidolon bat coronavirus/Kenya/KY24/2006] | ADX59468 |
|  | M | 71.43 | 71.23 | 71.29 | 91.98 | membrane protein [Bat coronavirus] | YP\_009824992 |
|  | N | 61.62 | 61.73 | 66.99 | 87.23 | nucleocapsid protein [Eidolon bat coronavirus/Kenya/KY24/2006] | ADX59470 |
|  | NS7a | 53.28 | 2.4† | 0.4† | 1.5† | hypothetical protein ORFx [Bat coronavirus] | AWV67068 |
|  | NS7b | 0† | 2† | 0.5† | 2† | hypothetical protein [Bat coronavirus HKU9] | ADM33571 |
|  | NS7c | None | 28.57 | 0† | 45.89 | hypothetical protein ORFy [Eidolon bat coronavirus/Kenya/KY24/2006] | ADX59472 |