**Title**

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

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**Keywords:**

*Nobecovirus, bat-borne coronavirus, recombination, zoonosis, Madagascar*

**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.* These novel *Nobecoviruses* 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 both viruses*.* Given the frequency with which coronaviruses, including Nobecoviruses, are known to recombine, these findings emphasize the need for more extensive coronavirus surveillance among wild bats in Africa to document the availability of viral sequences capable of infecting human hosts. Madagascar offers a unique phylogeographic nexus of bats and viruses with both Asian and African phylogeographic origins, offering opportunities for unprecedented mixing of viral groups. As bats are consumed widely across the island for subsistence, understanding the landscape of potentially zoonotic coronavirus circulation will be essential to mitigating 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 instead of bats (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), partly because many CoVs utilize well-conserved cell surface receptors to gain entry into 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) to do the same (35). Sarbecoviruses which cluster phylogenetically adjacent to ACE2-using lineages have been recently described in Kenyan *Rhinolophid* bats (36,37), highlighting the need for more intensive coronavirus surveillance in Africa. Because CoVs are notoriously inclined towards recombination—with other CoVs, or more rarely, with other viral groups—there is concern that naturally circulating CoVs presently unable to infect humans may acquire this ability in the future. Indeed, recombination has been implicated in many cross-species coronavirus emergence events (including zoonoses) (26,38–41), emphasizing the importance of widespread surveillance in characterizing the landscape of future zoonotic risks. Several factors, which have been reviewed at length elsewhere (31,42,43), contribute to the CoV affinity for recombination, including a large genome size supported by a unique proofreading mechanism in the CoV 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 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 across the island (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* (29,55). Previous studies have demonstrated that this latter *Betacoronavirus* RNA clusters with subgenus *Nobecovirus* (29,55); *Nobecoviruses* have been previously described in Pterodidae fruit bats across Asia and in both East (Kenya) and West (Cameroon) 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), recombination is a distinct possibility in the Madagascar CoV system. Though no *Rhinolophus* spp. bats, the typical host for ACE2-using *Sarbecoviruses*, inhabit Madagascar, the island is home to several species of bat in family Hipposideridae, which host the closely-related 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 tourist visitation sites (63–66). In addition to natural CoV diversity circulating in Malagasy bats, the Embecoviruses, HCoV-OC43 and HCoV-HKU1, and, more recently, the Sarbecovirus, SARS-CoV-2, are known to circulate widely among human hosts in Madagascar (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, assessing their past and future capacity for recombination and relatedness to previously described Nobecoviruses from Asia and other parts of Africa.

**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 (302), throat (143), and urine (196) swabs samples in the Virology Unit at Institut Pasteur of Madagascar, with each sample corresponding to a unique individual from the field dataset. 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 was stored 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*

*IDSeq*

*Offline BLAST Analysis*

*Phylogenetic Analysis*

1. *Full-genome phylogeny*
2. *RdRp phylogeny*
3. *Amino Acid phylogeny*

*Recombination Analysis*

*Nucleotide Sequence Accession Numbers*

**Results (cara)**

287 bats from 3 species were captured and sampled over one year from 2018-2019: P. rufus (n=44), *E. dupreanum* (n=146), and *R. madagascariensis* (n=95) (Figure 1). Urine samples, while taken, did not have any coronavirus hits. Of fecal samples, the breakdown of coronavirus prevalence was as follows: *P. rufus* (n=4/44, 9%), *E. dupreanum* (n=18/146, 12.3%), and *R. madagascariensus* (n=8/95, 8.4%) (Figure 1). Finally, of the coronavirus positive samples, the adult/juvenile breakdown was as follows: *P. rufus* (n=2 juvenile, 2 adult), *R. madagascariensis* (n=0 juvenile, 8 adult), and *E. dupreanum* (n= 5 juvenile, 13 adult).

GAM modeling to explore disease ecology of coronaviruses in *E. dupreanum*, *R. madagascariensis*, and *P. rufus* was plotted. *P. rufus* coronavirus prevalence appears to drop in anticipation of the dry season in Madagascar. The same pattern, although not as pronounced can be observed for *R. madagascariensis*. However, E*. dupreanum* coronavirus prevalence did not change much over time and over seasons. There is a nonsignificant rise in coronavirus prevalence around April in all three bat species that slowly tapers off into the dry season, then rises again going into January. The three species have similar breeding seasons (around April-May) and annual birth pulses (around October)46.

Paragraph about seasonal dynamics

Paragraph about phylogeny+RdRp

Comment about juveniles versus adults?

**Discussion**

Organize as:

1. Two novel Nobecos, cluster with Asian clades (Pteropus) and African (Rousettus) but evidence of recombination in S, N, NS7 genes
2. No evidence of orthereovirus insertion, suggests this strain may be limited to Asia. In fact, we can define four clades of Nobecoviruses broadly: HKU9, Eidolon helvum, GCCD1, and BatCoV92/GX2018.
3. Following on above, P. rufus does have extra genetic material between M and N, as does BatCoV92/GX2018, suggesting a dynamic region of the genome that could be a site for future recombination or acqusistion of new genes
4. Serious concern would be acquisition of S sequences enabling human cell entry. No known Sarbecoviruses on the island that could enable this but there are Merbecobivurses and M. jugalaris coroosts with Rousettus
5. Probably bigger concern is spillback and additional genetic material for SARS-CoV-2 which is widespread in Mada
6. All the seasonality stuff and importance of longitudinal studies
7. A plug for the importance of full genomes – only a handful of Nobeco genomes out there

We have described three novel nobecovirus sequences, most notably from R. madagascariensis, a bat host that had previously not been identified as a competent coronavirus host41. The average prevalence of 10% is comparable to sample efforts in other countries, indicating that there is an endemic level of coronaviruses circulating throughout Madagascar28,29. The novel nobecoviruses isolated are closely related to nobecoviruses isolated from China and Singapore, also mostly from *Rousettus spp.* (Figure 3A). The RdRp clustering also shows close homology with African coronavirus strains, along with further showing relation to Asian coronavirus strains (Figure 3B). Seasonality modeling of coronavirus prevalence revealed little data to correlate infection data to bat breeding seasons and annual birth pulses, so more data is needed to correlate the time of year the sample was collected to food availability, depending on the species’ diet (Figure 2). Stress in these bat species my also dictate coronavirus success in these hosts, as stress can dampen the immune response46. Multi-year longitudinal studies will be necessary to untangle these interactions. A next logical step would be to getting a full genome coronavirus from *E. dupreanum*.

It is known that these endemic species of bats can co-roost in the same habitats; *R. madagascariensis* and *E. dupreanum* roost in caves, whereas P. rufus roosts in trees46. While no full genomes were isolated from *E. dupreanum*, the RdRp panel indicates that *E. dupreanum* and *R. madagascariensis* coronaviruses cluster more closely than either individually with *P. rufus.* This could suggest that recombination events may take place between occasional co-roosting species, as shown before in other bat coronavirus sampling studies46,50. In China, co-roosting bat species from one mine shaft yielded samples of a new *Sarbecovirus*, along with other novel *Betacoronaviruses*50. Recombination events have been observed frequently with coronavirus; there is evidence that SARS-CoV-2 emerged from a stepwise recombination series over time42,51–55. One study found a coronaviruses in Africa that appears to be an intermediate step between SARS-CoV-1 and SARS-CoV-2 in terms of similarity in the receptor binding domain, but without the ability to bind ACE256. ACE2 usage is well described in many coronaviruses from Asia, but more focus should be on bridging the gap in this knowledge from other countries52,56.

A previous coronavirus sampling study of Madagascar fruit bats found viruses in *P. rufus* and *E. dupreanum*, but not *R. madagascariensis*, although they only detected one virus in *E. dupreanum*29. Most of their sampling was also within a one year span, and mostly restricted to one region, which could explain the skewing of positive samples toward one bat species, but still resulted in an overall prevalence of 4.5%29. Another study of coronavirus sampling in the West Indian Ocean provided more information about prevalence in Madagascar (around 5%) with a larger sample set that is more ubiquitously spread about the island, but also showed that the islands sampled have similar coronavirus prevalence to that of Africa28. Additionally, it is suggested that the dominant evolutionary mechanism for coronaviruses in this region is due to co-evolution, possibly supplemented by host switching in co-roosting situations28. In contrast to other Madagascar bat sampling studies, our work indicates a general prevalence of 10% among the three bat species. While slightly higher, it is still comparable to coronavirus prevalence in the region28,29. Pathogen spillover from bats is also dictated by ecological factors such as seasonality, waning immunity, and other stressors such as nutrition access and breeding seasons37,46. In our study, the highest prevalence of coronaviruses occurred between March-April, leading up to the breeding season for the three bat species.

Data on human risk from these coronaviruses is lacking. Bats come into contact with humans on Madagascar through habitat destruction along with through hunters, several bat species are consumed39,40,46,47. Close contact with roosting habitats such as caves not only puts a human at risk of direct bat contact, but also with guano. In addition to longitudinal sampling of bats, it would be beneficial to supplement this data with antibody studies from local human populations such as hunters to assess zoonotic risk, with a particular focus on coronaviruses along with other pathogens of interest such as henipaviruses that are shown to replicate in these species discussed46. With how ubiquitous bats are, it is important to recognize the risk while also understanding that they are important members of many ecosystems, and protection from habitat loss and encroachment would go a long way in preventing unnecessary human/bat interactions.

Contribution to the Field Statement:

**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*.

**Author Contributions:**

**Funding:**

**Data Availability Statement:**

**References**

Figure Legends

**Fig 1**: Map of sampling sites for P. rufus, E. dupreanum, and R. madagascariensis. Circles are in log scale and sorted by CoV negative or positive and adults or juvenile, CoV prevalence in P. rufus, E. dupreanum, and R. madagascariensis over time

**Fig 2:** Genome structure of isolated full genomes, TRS table in word format

**Fig 3:** Full genome+RdRp phylogeny

**Fig 4:** Simplot+bootscan to look for recombination

**Supplementary figs:** BLAST table, phylogenies of N, S, M, E

**Table 1:** TRS locations

**Table 2:** BLAST results

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