**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.* 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 associated with bat hosts1–4; the remaining CoV genera, *Gammacoronavirus and Deltacoronavirus,* are typically hosted by birds (1). The *Betacoronavirus* group can be further broken down into bat-associated subgenera *Sarbecovirus* (hosted by *Rhinolophus* spp.5,6), *Merbecoviruses* (hosted by *Pipistrellus* and *Tylonycteris* spp.7–9), *Nobecovirus* (hosted by *Eidolon* and *Rousettus* spp.10–12), *Hibecovirus* (hosted by *Hipposideros* spp.13,14), and non-bat associated subgenus *Embecovirus,* which is associated with humans, rats, and bovine species15,16. All but the latter have been associated with bat hosts15,16. Since the SARS-CoV-1 epidemic, there has been more interest in surveying potential hosts of coronaviruses and contributing new virus sequences to public databases, with most effort focused on sampling bats from Asia17–24. As SARS-CoV-1 and SARS-CoV-2 have emerged from this county, most of the sampling effort has been based here, and it has only been recently that a more concerted effort has been underway to survey the landscape of coronaviruses that reside in bat populations in countries like Africa and Europe25–33.

Bats are ubiquitous mammals across all continents except Antarctica, and frequently their habitats overlap with the habitats of other mammals and humans34–37. Deforestation, urbanization, wet markets, and resource usage such as entering caves for guano and hunting bats for food all are activities initiated by humans that put them in contact with bat populations more frequently and thus contribute to a risk of zoonotic transmission34–40. Novel coronaviruses have been well described in Asia, especially in *Rhinopolus spp*23,41, although recent surveying has found coronavirus diversity in African and European bats as well25,26,32,33,42–45.

Madagascar is an island country, adjacent to Mozambique in southwest Africa. Isolated from the rest of Africa or other nearby countries, Madagascar hosts about 40 different species of bats, half of them endemic populations46. With many bat species and interactions with humans in Madagascar (such as hunting for bushmeat, habitat encroachment)40,46–48, it is important to sample bat populations for potential coronaviruses that may one day become zoonotic34,36,37,39,43,49.

Here we contribute three novel nobecovirus full genomes from Madagascar fruit bat fecal samples (two from *R. madagascariensis*, one from *P. rufus*). These additions add to the landscape of sequences that exist to survey the populations of coronaviruses that are endemic to these island bats and further serve to understand the zoonotic risk, especially since *R. madagascariensis* had not been previously identified as a potential coronavirus host.

**Materials and Methods**

Study sites bat sampling

RNA extraction

Viral amplification and detection

Phylogenetic analysis

Taxonomic analysis

Phylogenetic and 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

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Comment about juveniles versus adults?

**Discussion**

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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