Novel bat astrovirus strain from *Rousettus madagascariensis* sheds light on the evolutionary history of bat astroviruses

Molecular detection of whole genome sequences of novel astroviruses in Malagasy bats

INTRODUCTION

Bats (order: Chiroptera) are an extremely diverse mammalian order that in recent years have been posited as reservoirs of many of the world’s most virulent emerging viruses 1,2. This disproportioned representation is thought to be due to a number of reasons, including immune system modifications due to the evolution of flight, long lifespans, and dense roosting aggregations that facilitate intra- and inter- species transmission 1,3,4. Despite their importance as reservoirs, thorough surveillance of the viruses hosted by bats has been restricted to few species and locations.

The island of Madagascar provides an important case study for viral surveillance to test the presence and evolutionary history of bat viruses and investigate their zoonotic potential. Madagascar hosts 46 bat species with nearly 80% endemism, the result of a unique biogeographical history5,6. This isolation has also led to the coevolution of extraordinarily diverse viruses within Malagasy bats7,8. Moreover, high human-bat contact rates due to hunting, particularly of large fruit bats, across the island increase the potential for zoonotic spillover9.

A key understudied family of viruses are Astroviruses (AstVs) (family: *Astroviridae*). Astroviruses are positive-sense, single-stranded RNA viruses with a remarkable diversity of hosts, grouping broadly into two genera: *Avastrovirus,* which infect avian hosts, and *Mamastrovirus,* which infect mammalian hosts10. These hosts include humans, in which astroviruses are thought to be responsible for 2-9% of all acute non-bacterial gastrointestinal infections in children11. There is substantial evidence of past zoonotic transmission of astroviruses11–15, thought to be facilitated by high diversity and recombination events, and clear gaps in sampling and evolutionary history highlight the need to improve our understanding of astroviruses.

Astroviruses have been detected with notable presence and remarkable diversity in bats, though these values range widely depending on the study design, species, and location16–20. Differing phylogenetic analyses reveal both strong and weak host- and geographic- clustering, with some bat astroviruses grouping more closely with members of the avian *Avastrovirus* genus. The detection of high astrovirus diversity detected within members of the same population suggests the simultaneous circulation of multiple strains, perhaps driven by the co-roosting of multiple species in dense aggregations and again highlighting the importance of bats as drivers of virulence. These insights, however, have been largely based on PCR data from few species of bats, limiting the potential for more robust evolutionary analysis. Very recently, metagenomic next-generation sequencing has resulted in the detection of five full-genome bat astroviruses. However, limited analysis has been done with these sequences and representation among bat species and geographic location remain extremely limited.

This paper presents results on metagenomic next-generation sequencing (mNGS) detection and characterization of astroviruses sampled from three species of endemic fruit bats from Madagascar*: Pteropus rufus,* *Rousettus madagascariensis,* and *Eidolon dupreanum*. We present and characterize a novel full-length genome from *Rousettus madagascariensis* and examine the evolutionary history of bat astroviruses from the Southwest Indian Ocean Region.

METHODS

**Sample collection and processing**

Astrovirus infections were identified from a dataset of viruses detected in samples from a long-term longitudinal sampling of fruit bats across Madagascar. Details on bat sampling and sequencing protocol can be found in previous work8; here we will only give a brief overview.

Between 2018 – 2019, monthly bat captures were taken out at four species-specific locations: Ambakoana roost (-18.513 S, 48.167 E, *Pteropus rufus)*; Angavobe cave (-18.944 S, 47.949 E, *Eidolon dupreanum)*; Angavokely cave (-18.933 S, 47.758 E, *Eidolon dupreanum)*; Maromizaha cave (-18.9623 S, 48.4525 E, *Rousettus madagascariensis).* Bats were identified to species, sex, and age (adult vs juvenile), and throat, fecal, and urine samples were taken.

In total, RNA from 285 fecal, 143 throat, and 196 urine swab samples was extracted, then prepped into libraries and submitted for Illumina sequencing (see supplemental table). Raw reads from Illumina were filtered and assembled on the CZID bioinformatics platform (v3.10 NR/NT 2019-12-01). Samples were marked positive for astrovirus infection if at least two contigs with an average read depth >2 reads/nucleotide were assembled that showed significant nucleotide or protein BLAST alignment to astroviruses present in NCBI NR/NT database (v12-01-2019).

**Genome Annotation and BLAST**

We annotated all novel Astrovirus sequences by aligning them to annotated homologs published on NCBI and visually identifying open reading frames, using Geneious Prime (v08-18-2022).

**Phylogenetic Analysis Overview**

To perform phylogenetic analysis, we combined Astrovirus sequences identified in this study with those publicly available on NCBI. We carried out three major phylogenetic analyses, building (a) a full-genome *Mamastrovirus* maximum (ML) likelihood phylogeny, (b) a time-resolved Bayesian phylogeny corresponding to a selection of full genome *Mamastrovirus* sequences available on NCBI Virus, and (c) a *Mamastrovirus* ML phylogeny corresponding to a conserved 410 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene encapsulated in the AstV ORF1b. Detailed methods for the construction of each phylogeny are available on GitHub (see supplementary information).

**Sequence Compilation**

Our full genome ML phylogeny consisted of one novel full length *Mamastrovirus* sequence, 41 unique NCBI *Mamastrovirus* sequences, and one full length *Avastrovirus* sequence as an outgroup, for a total of 43 sequences. We compiled the NCBI sequences through three queries: 1) all complete RefSeq Genomes under Virus: *Mamastrovirus* (taxid:249588) and Virus: *unclassified Mamastrovirus* (taxid:526119) greater than 6,000 bp (36), 2) *Mamastrovirus* nucleotide genomes under Virus: *Astroviridae* (taxid:39733) and Virus: *unclassified Astroviridae* (taxid:352926) with Host: *Chiroptera (bats)* (taxid:9397) over 6,000 bp (2), and 3) manual searching of *Mamastrovirus* nucleotide genomes >6,000 bp identified in the literature (3).

After building our full genome ML tree, we subselected one monophyletic clade containing all known bat sequences and their closest relatives for the Bayesian time-tree analysis. This included a total of 17 sequences: 16 from NCBI virus and our novel *Rousettus madagascariensis* sample.

Our *Mamastrovirus* RdRp ML phylogeny consisted of an overlapping subset of a 410 bp fragment in the center of the RdRp region of our novel full length *Mamastrovirus* sequence, 122 unique NCBI *Mamastrovirus* sequences, and one Avastrovirus RdRp fragment as an outgroup, for a total of 124 sequences. NCBI sequences were restricted to those from bat hosts sampled in the Southwest Indian Ocean (SWIO) region. They were compiled through one query: Virus: Astroviridae (taxid:39733), Host: Chiroptera (taxid:9397), and Geographic Region: Madagascar (64), Mozambique (31), and Reunion (27). Sequences were confirmed to be RdRp fragments via alignment and relevant metadata was confirmed though source literature.

**Alignment and Substitution Model**

Following dataset compilation for each phylogenetic analysis, sequences were aligned via the MAFFT21 (v7.450) algorithm in Geneious Prime (v 2022-08-18) using default parameters. Alignments were visually examined and trimmed to conserved regions. We then used Modeltest-NG22 (v0.1.7) to determine the best fit nucleotide substitution for each alignment. All sequences, subsets, and alignments are available in our open-source GitHub repository (see supplementary information).

**Phylogenetic Tree Assembly**

Both the full genome and RdRp ML trees were constructed in RAxML-NG23 (v1.1.0), using the best nucleotide substation model from Modeltest-NG. Following best practice recommendations in RAxML-NG, twenty ML inferences were made, followed by bootstrap replicate trees inferred using Felsenstein’s method24. The MRE-based bootstrapping test was performed every 50 replicates, and bootstrapping was terminated when the diagnostic result was below the threshold value. Support values were compiled onto the best-scoring tree.

The Bayesian time tree was built with full-genome astrovirus sequences from the clade containing all bat full-genomes as identified from the maximum-likelihood tree using BEAST225 (v2.6.7). We used a Bayesian Skyline Coalescent Model with a strict lognormal clock rate with mean 0.0001 and all other priors as default8. Sampling date for each sequence was inferred from NCBI ‘Collection Date’ or though reading source literature; if day and month were not available the sampling date was set to July 15th. Markov Chain Monte Carlo (MCMC) chains were run for >700,000,000 iterations and terminated when we identified convergence using TRACER (v1.7), with 10% burn-in. We used TreeAnnotator (v2.6.3) to examine mean posterior densities at each node.

All phylogenies were visualized in RStudio (v2022.07.01), using the package ‘ggtree’26.

**Nucleotide Sequence Accession Numbers**

One annotated full-length genome sequence from *Rousettus madagascariensis* was submitted to NCBI and assigned accession number: XXXXXX.

RESULTS

**Astrovirus Detection**

RNA from 285 fecal, 143 throat, and 196 urine swab samples from was prepped into libraries and submitted for Illumina sequencing. We defined astrovirus positive samples as all sequences for which a minimum of two AstV contigs were assembled in CZID with an average read depth >2 reads/nucleotide. We detected AstV positives samples in 4/285 (1.4%) fecal samples and 7/196 urine samples (3.57%). None of the 143 throat swabs assayed demonstrated evidence of AstV infection.

Detection varied slightly between species, with 1/44 (2.27%) *Pteropus rufus*, 8/145 (5.52%) *Eidolon dupreanum*, and 2/96 (2.08%) *Rousettus madagascariensis* positives detected, but these differences were not significant (χ2=2.104, P=0.3492). Because one bat species is sampled per site, this indicates that sampling between sites was likewise not significantly different.

Juvenile vs adult prevalence also did not vary significantly: 1/15 (6.66%) vs. 0/29 (0%) for *P. rufus* (χ2=1.941, P=0.1635), 1/13 (7.69%) vs. 7/132 (5.30%) for *E. dupreanum* (χ2=0.122, P=0.7264), and 1/13 (7.69%) vs. 1/83 (1.20%) for *R. madagascariensis* (χ2=2.271, P=0.1318) (Figure 1).

Diagram

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Figure X: Map of sampling sites for *P. rufus*, *E. dupreanum*, and *R. madagascariensis* in the districts of Moramanga and Manjakandriana, Madagascar (*P. rufus:* Ambakoana roost; *E. dupreanum*: Angavobe/Angavokely caves; *R. madagascariensis*: Maromizaha cave). Pie charts correspond to astrovirus prevalence in juveniles vs. adults across all three species: 1/15 (6.66%) vs. 0/29 (0%) for *P. rufus*, 1/13 (7.69%) vs. 7/132 (5.30%) for *E. dupreanum*, and 1/13 (7.69%) vs. 1/83 (1.20%) for *R. madagascariensis*. Pie circle size corresponds to sample size on a log-10 scale.

**Genome Annotation and Similarity Analysis**

One near-full length Astrovirus contig with high coverage was recovered from a male, juvenile *Rousettus madagascariensis* bat*,* 6,593 bp in length. By aligning this sequence to annotated full genome *Mamastroviruses* from NCBI, we successfully annotated ORF1a, ORF1b, which contains the RdRp region, and ORF2. An additional near full length Astrovirus contig was recovered from the same individual, but the coverage was low and so it was disregarded (see supplement).

All other astrovirus detections aligned within the ORF2 region, which does not contain coding regions that have been the target of PCR.

BLAST analysis of the near-full genome against all available partial and full astrovirus sequences in NCBI indicated that this *R. madagascariensis* sequence is highly divergent, demonstrating only 75.91% - 79.63%. All top blast associations were in other chiropteran species (see supplement).

This divergence is supported by nucleotide and amino acid similarity plots comparing this novel sequence to all full genome bat Astrovirus sequences from NCBI (Figure X). Using the novel near-full sequence as a query (F\_MIZ141\_1), we see low similarity across the entire genome, with all other bat sequences cluster together at a range of average nucleotide similarity from 28.18% - 40.28% (Fig X A) and average amino acid similarity from X-X (Fig X B).

Graphical user interface, application

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Figure X: Similarity plots comparing all chiropteran full genome astrovirus sequences to the novel Madagascar bat astrovirus sequences for A) nucleotide sequences, and B) amino acid sequences. Query sequence is F\_MIZ141\_1, comparison sequences are MG693176 *(E. helvum)*, MZ218054 *(M. daubentonii 1),* MZ218053 *(M. daubentoniid 2),* MN832787 *(M. daubentoniid 3),* MT734809 *(Myotis yumanensis).*

**Phylogenetic Analysis**

Diagram

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Figure X: Maximum likelihood phylogeny of full genome *Mamastrovirus* sequences (RAxML-NG, TVM+I+G4). Bootstrap values computed using Felsenstein’s method24 are visualized on tree branches. Tip labels include NCBI taxon ID, strain, host species, location of collection, and year of collection. Tip points are colored by order of mammalian host, and shaped by novelty, with Madagascar sequences labeled with triangles. The novel astrovirus sequence presented in this paper is highlighted in yellow. Tree is rooted by a turkey *Avastrovirus* (NC\_002470) and a divergent Porcine *Mamastrovirus* (NC\_023636). Branch lengths are scaled by nucleotide substitutions per site, corresponding to the scalebar.

Chart

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Fig X: Bayesian phylogeny of full genome *Mamastrovirus* sequences generated from >700,000,000 steps under a Bayesian Skyline Coalescent Model (TVM+I+G4). Node color represents mean posterior estimates averaging over all steps with 10% burn-in (see scale bar on left). Tip labels include NCBI taxon ID, strain, host species, sampling location, and year of sample, and are colored by strain, with our novel astrovirus sequence highlighted in yellow.

***Mamastrovirus* full-genome evolutionary history**

The full-genome maximum likelihood tree resolves two distinct clades, with a single Porcine astrovirus grouping with the *Avastrovirus* outgroup. No host order demonstrates monophyly, with high support in most cases. Bat astroviruses are a paraphyletic group basal to the clade containing astroviruses from Artiodactylia, Carnivora, and Primates (in this case, humans).Our novel astrovirus groups most closely with a sample from a Cameroonian *Eidolon helvum* and fall out more recently derived than the insectivorous bat astroviruses*.*

**Bayesian time-tree**

The Bayesian time-tree agrees with topology from the maximum likelihood tree, except reorders the basal group to instead be the clade containing the mouse, *Myotis yumaneisis,* and *Myotis daubentoniid* samples. With high posterior support, it identifies that the clade containing bat astroviruses diverged ~30,000 years ago, that the clade containing our novel sequence and that from *E. helvum* diverged ~25,000 years ago, and that our sequence diverged from *E. helvum* ~15,000 years ago.

Diagram, schematic

Description automatically generated

Figure X: Maximum Likelihood phylogeny of a 410bp fragment of *Mamastrovirus* Orf1b (RdRp) from Southwest Indian Ocean (SWIO) bat hosts (RAxML-NG, TVM+I+G4). Bootstrap values computed using Felsenstein’s method (CITE) and those >30 are visualized on tree branches. Tip labels include NCBI taxon ID, host family, and host species. Tip points are colored by sub-order and shaped by country of origin. The novel astrovirus sequence presented in this paper is highlighted in yellow. Tree is rooted by a Turkey *Avastrovirus* (NCBI taxID: NC\_002470) and a divergent bat *Mamastrovirus* (NCBI taxID: MZ614426). Branch lengths are scaled by nucleotide substitutions per site, as indicated by the scale bar.

**RdRp SWIO phylogeny**

AstV sequences in the RdRp phylogeny group according to host phylogeny over geography of sampling site, a pattern which holds across both order and family across all sampling locations. Almost all Yinpterochiropteran *Mamastrovirus* sequences fall into a monophyletic group, and Yangochiropterans appear in multiple clades basal to this group. Within Yangochiropterans, Molossids and Miniopterids cluster tightly together regardless of location.

There are a few notable exceptions to this pattern. One is sequences from the bat family Nycteridae, which fall both within Molossids and as basal to the Yinpterochiropteran clade. Another is representatives from Vespertilionidae, which group more closely with Yinpterochiroptera than Yangochiroptera. Finally, there appears to be an overall trend of Yinpterochiropteran samples being more likely to fall within the Yangochiropteran clade than vice versa. Our novel *Rousettus madagascariensis* sample is one example of these—instead of grouping within the Yinpterochiropteran clade or even closely with the other Madagascar *R. madagascariensis* sample, it falls out within the Molossidae astroviruses.

DISCUSSION

This paper utilizes mNGS sequencing to detect and characterize astroviruses from three endemic species of Malagasy fruit bats, *Pteropus rufus, Rousettus madagascariensis,* and *Eidolon dupreanum.*

**Detection**

Astroviruses were detected in all species across all sampled locations. This work is the first detection of astroviruses from two endemic species of Malagasy fruit bat: *Eidolon dupreanum* and *Pteropus rufus*. In combination with Lebarbchenon et al, astroviruses have now been detected in 7 species of Malagasy bat. While detected across all samples, our infection was low, in all cases below 8%, however, this level of infection has been described in other bats, particularly Pteropodids27,28. Other studies, however, detect astrovirus infection as high as 51%29, but our infection data lends to a growing trend that it appears that insectivorous bats host higher levels of astrovirus infection. We see a slight difference between adults and juveniles, no difference in seasonality. Seasonality has been detected in some studies, not in others27. Lebarbchenon et al detected astrovirus in 2/41 sampled *Rousettus madagascariensis* (4.8%).

We detected more astrovirus infections via urine samples than fecal samples.

(7/196 urine samples (3.57%) vs 4/285 (1.4%) fecal samples). Although astroviruses are known to transmit fecal-orally, they have been detected in bat urine across multiple studies30.

Differences in detection could be due to sampling bias or could reflect variation in AstV infection. Because these species and locations are part of a long-term longitudinal study, there is strong future potential to further develop patterns of AstV variation in seasonality, locations, and hosts. However, our consistent detection of astroviruses across bat species and geographic locations lends support to bats as likely reservoirs of AstV’s.

Our detection of a whole genome allowed for valuable insight into the genetic diversity of this novel astrovirus strain. Similarity analysis of our full genome with all other bat astrovirus full genomes support its strong diversity, with nucleotide similarity often less than 50% and amino acid similarity often less than 20%. This is in line with other studies demonstrating the extraordinary divergence of Madagascar bat viruses, likely from the long isolation the island has experienced8. Interestingly, the other Pteropodid genome from *Eidolon dupreanum* does not emerge as more closely related to our *R. madagascariensis* genome than to genomes from *Myotis* spp., an insectivorous bat. Based on the ICTV’s definition of a new strain as defined by being X% different in the capsid region, our genome is a new full strain.

BLAST analysis identified a second near full genome astrovirus sequence from the same individual. Coverage from this sample was low (see supplement) thus it was not included in phylogenetic analysis—however, its presence suggests the possibility of coinfection with multiple astrovirus strains. Astrovirus coinfection has been detected in bat species, including fellow Pteropodid *Eidolon helvum*31*.*

While the detection of a whole genome provided valuable insight, all other fragments detected through mNGS did not contain the RdRp region that has been targeted in all previous PCR detection of bat astroviruses, thus restricting their ability to be used in evolutionary analysis.

**Full genome phylogeny**

To place our novel astrovirus strain within the evolutionary history of *Mamastrovirus*, we built a full genome maximum likelihood phylogeny using all available bat full genomes and all available reference full genomes from other host species. Our phylogeny postulates two *Mamastrovirus* clades, placing all five bat astroviruses within the same clade. In the clade, however, they are not monophyletic, nor do they follow phylogenetic relationships. Additionally, bat astroviruses are basal to several human astroviruses. This supports the known propensity for astroviruses to frequently cross species boundaries, as well as supporting a history of zoonotic transfer. Much more extensive sampling to increase the number of whole genome bat astroviruses is clearly necessary to resolve relationships.

To investigate the timing of the origination of the currently known bat astrovirus strains, we built a Bayesian time tree of the clade containing all known bat astroviruses. This Bayesian tree resolves the same relationships as the maximum likelihood tree, except it changes which clade is basal. The tree estimates a deep evolutionary history for this Mamastrovirus clade, with the MRCA of bat astroviruses diverging ~30,000 years ago, and the MRCA of our novel bat astrovirus diverging from that from *Eidolon helvum* ~15,000 years ago. Considering Madagascar’s isolation from the African continent for ~160 million years and the divergence of the Rousettus genus ~20 million years ago32, this would indicate recent viral genetic exchange between bats from Madagascar and the African continent. Recent publications investigating the divergence times of other viruses from Malagasy bats support this hypothesis8.

**RdRp SWIO phylogeny**

To investigate the biogeographical evolutionary history of bat astroviruses, we built an RdRp tree with all bat *Mamastrovirus* samples from the Southwest Indian Ocean Region. In all, we had representatives from X species across three sampled locations: Madagascar, Mozambique, and Reunion Island. Largely, astrovirus samples grouped by phylogenetic relationship of bat family than by sampling location, following the evolutionary separation of Yinpterochiroptera and Yangochiroptera33. This is in contrast to many other astrovirus studies, where host restriction was found to be low or nonexistent7,28,34–37.

Within the Yinpteropchiropterans, Rhinocyterid astroviruses mixed with Hipposiderids, both with representatives from Madagascar and Mozambique. Numerous papers have established them as sister families6,33, thus there genetic similarity to each other makes cross-transmission between them more likely.

Within the Yangochiropterans, the two most sampled families grouped separately: Minopteridae and Molossidae. Molossid samples did not group within the other microbats, and instead fell out in numerous clades basal to all other samples. Paraphyly has been detected in Molossid species33, and thus may promote paraphyly in their viruses through coevolution. This host restriction, however, may be confounded with sampling location, since only Reunion Island Molossids and Madagascar Miniopterids were represented. Interestingly, Molossids from Madagascar have been found to have low to no prevalence of astrovirus depending on sampling location7,38.

There were a few notable exceptions to the family and sub-order level host restriction. Nycterid samples fell out in two disparate locations across the phylogeny: one cluster within the Molossids and another as basal to the Yinpterochiropteran clade. Vespertilionids, with a sample size of only two, were placed within the Yinpterochiropteran clade, most closely related to Rhinonycterids.

Pteropodid samples did not group together or fall out in their accepted phylogenetic placement as basal to Yinpterochiropterans6, despite all being sampled from the same endemic Madagascar species, *Rousettus madagascariensis*. All three samples fell out on very different places on the phylogeny—one within Yinpterochiroptera most closely related to a Rhinocyterid sample, one within the Yangochiropteran Miniopterids, and our novel one within the Yangochiropteran Molossids.

It appears from this phylogeny that Yinpterochiropteran astroviruses appear more often within Yangochiropteran clades than vice versa. While there are only 3 microbat samples within the megabat cluster, and they all fall out basally, there are 8 megabat samples found within microbat clusters, and they are widely dispersed throughout the phylogeny.

Close phylogenetic relationships between viruses hosted by different species indicates cross-species transmission. In the case of bats, this is promoted by several features of bat behavior and biology, including immune system modifications due to the evolution of flight, long lifespans, and dense roosting aggregations that facilitate intra- and inter- species transmission 1,3,4. However, the presence of more Yinpterochiropteran virus samples nested within Yangochiropteran virus samples appears to suggest that the likelihood of transmission between the two groups may not be equal.

It should be noted, however, that many of these basal nodes have weak support values, therefore our confidence in these relationships isn’t particularly strong. Further sampling is necessary to help elucidate these patterns.

CONCLUSION

DATA AVAILABLITY STATEMENT

The sequences presented in the study are deposited in NCBI, accession number: XXXX.

ETHICS STATEMENT

The animal study was reviewed and approved by UC Berkeley Animal Care and Use Committee and Madagascar Ministry of Forest and the Environment under guidelines posted by the American Veterinary Medical Association.

AUTHOR CONTRIBUTIONS

CB conceived of the project and acquired the funding, in collaboration with J-MH, PD, JD, and CT. Field samples were collected and RNA extracted by AA, SA, AG, HR, TR, NR, and CB. AK led the mNGS, with support from VA, HR, TR, and CB. SH and CB analyzed the resulting data and co-wrote the original draft of the manuscript, which all authors edited and approved.

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SUPPLEMENTARY MATERIAL

Github Link

Table 1: illumina sequencing input (sample type, sample id)

Table 2: blast results

Coverage plot of discarded astrvirus full genome

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