

Rapid evolution of SARS-CoV-2 in domestic cats

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Abstract

SARS-CoV-2 (SARS2) infection of a novel permissive host species can result in rapid viral evolution. Data suggest that felids are highly susceptible to SARS2 infection, and species-specific adaptation following human-to-felid transmission may occur. We employed experimental infection and analysis of publicly available SARS2 sequences to observe variant emergence and selection in domestic cats. Three cohorts of cats ($N = 23$) were inoculated with SARS-CoV-2 USA-WA1/2020 or infected via cat-to-cat contact transmission. Full viral genomes were recovered from RNA obtained from nasal washes 1–3 days post-infection and analyzed for within-host viral variants. We detected 118 unique variants at ≥ 3 per cent allele frequency in two technical replicates. Seventy of these (59 per cent) were nonsynonymous single nucleotide variants (SNVs); the remainder were synonymous SNVs or structural variants. On average, we observed twelve variants per cat, nearly 10-fold higher than what is commonly reported in human patients. We observed signatures of positive selection in the spike protein and the emergence of eleven within-host variants located at the same genomic positions as mutations in SARS2 variant lineages that have emerged during the pandemic. Fewer variants were noted in cats infected from contact with other cats and in cats exposed to lower doses of cultured inoculum. An analysis of ninety-three publicly available SARS2 consensus genomes recovered from naturally infected domestic cats reflected variant lineages circulating in the local human population at the time of sampling, illustrating that cats are susceptible to SARS2 variants that have emerged in humans, and suggesting human-to-felid transmission occurring in domestic settings is typically unidirectional. These experimental results underscore the rapidity of SARS2 adaptation in felid hosts, representing a theoretical potential origin for variant lineages in human populations. Further, cats should be considered susceptible hosts capable of shedding virus during infections occurring within households.

Key words: SARS-CoV-2; felids; domestic cats; within-host variants.

Introduction

The devastating global effects of the coronavirus disease 2019 (COVID-19) pandemic have drawn attention to the relatively broad host species range of the SARS-CoV-2 (SARS2) virus. In addition to humans, known susceptible hosts include a wide range of wild and domestic animals that have been naturally or experimentally exposed to the virus. The variety of animal species infected by SARS2 highlights the importance of studying the virus in other susceptible hosts, with the goal of evaluating the potential for animals to act as disease reservoirs and mediate further viral evolution and to characterize the changes associated with cross-species coronavirus transmission.

Many species within the carnivore family *Felidae* are susceptible to SARS2, and transmissions from human-to-felid and felid-to-felid have been established (Garigliany et al. 2020; Gaudreault et al. 2020; Halfmann et al. 2020; Shi et al. 2020; Bosco-Lauth et al. 2020a; Braun et al. 2021; Hosie et al. 2021). Experimental exposures of domestic cats (*Felis catus*) to SARS2 have also been previously described (Gaudreault et al. 2020; Halfmann et al. 2020; Shi et al. 2020; Bosco-Lauth et al. 2020a;

Braun et al. 2021). Although most experimentally infected cats remained asymptomatic throughout infection, a range of respiratory and gastrointestinal signs have been reported in domestic cats naturally infected by their COVID-19-positive owners (Garigliany et al. 2020; Segalés et al. 2020; Curukoglu et al. 2021; Ferasin et al. 2021; Hosie et al. 2021; Keller et al. 2021; Klaus et al. 2021; Neira et al. 2021; Barroso-Arévalo et al. 2022; Zoccola et al. 2021). Infections of large numbers of captive wild felids have been documented throughout the pandemic (USDA APHIS 2022; Shu and McCauley 2017; McAloose et al. 2020; Bartlett et al. 2021; Fernández-Bellón et al. 2021; Karikalan et al. 2021; Mishra et al. 2021), with occasionally fatal consequences for some individual animals (Thebault, Reis 2021).

Recurring reports of SARS2-infected felids suggest that human-to-felid transmissions occur frequently, and infections of domestic household cats are likely unreported due to the lack of recognizable symptoms in most individuals. Several studies have aimed to estimate the incidence of domestic cat infections using serology. All but one study (Temmam et al. 2020) of cats living in households with known COVID-19 infection have detected antibodies in some proportion of cats, ranging from 3 per cent to

over 50 per cent of cats tested (Barrs et al. 2020; Calvet et al. 2021; Fritz et al. 2021; Hamer et al. 2021). An early study conducted in Wuhan, China, reported 14.7 per cent seropositivity in cats (Zhang et al. 2020), while other surveys of domestic cat populations have observed rates of infection, ranging from 0 to 5.8 per cent (Patterson et al. 2020; Villanueva-Saz et al. 2021), collectively, revealing the regularity of felid infections.

SARS2 genome sequences recovered from experimentally or naturally infected felids have led to the identification of some single nucleotide polymorphisms (SNPs), or consensus variants (present at >50 per cent frequency within a host), but no felid-adapted variants have been substantiated to date (Barrs et al. 2020; Braun et al. 2021; Hosie et al. 2021; Klaus et al. 2021; Bashor et al. 2021). In general, sequences recovered from naturally infected felids have closely resembled sequences recovered from their owners or zoo keepers. A smaller subset of experimental studies have characterized within-host viral diversity in domestic cats and reported cat-to-cat transmission of viral variants (Braun et al. 2021; Bashor et al. 2021). Cat-to-cat transmission was characterized in one study by a narrow bottleneck, with only 2–5 viral genomes transmitted (Braun et al. 2021), which is consistent with what occurs between humans (Lythgoe et al. 2021). However, both low-frequency and consensus variants were reliably transmitted, including variants under positive selection (Braun et al. 2021; Bashor et al. 2021). We have recently reported the rapid emergence of numerous variants in experimentally exposed cats, including mutations identical to or at the same genomic position as mutations characteristic of human SARS2 variant lineages (Bashor et al. 2021).

It is likely that SARS2 faces unique selective pressures when it ‘spills over’ into new hosts, which results in viral variant emergence and virus–host adaptation. The prevalence of SARS2 in felids draws attention to the possibility for spillback infections and novel variant transmission from felids to humans. Among species known to be highly susceptible to SARS2 and capable of shedding virus following infections, domestic cats have the most intimate contact with humans, with approximately 25 per cent of US households supporting an average of 1.8 cats (American Veterinary Medical Association 2017). Companion cats thus represent a unique concern among SARS2-susceptible nonhuman animals relating to their role in perpetuating infections or resulting in propagation of viral variants that may re-infect humans. Furthermore, repeated natural transmissions and host adaptation would support the potential establishment of a felid reservoir of SARS2.

In this study, we assessed SARS2 adaptation in felids through experimental exposure of three experimental cohorts of domestic cats to SARS2 and analysis of existing SARS2 sequences from cats. Experimental infection systems offer a unique opportunity to study controlled transmission and observe viral variant emergence and adaptation. Eighteen cats were directly inoculated, and five others were exposed through contact with one or more inoculated cats. A cohort of juvenile cats was exposed to low, medium, or high doses of SARS2. Nasal lavage samples were recovered 1–3 days post-infection (dpi), and full SARS2 genomes were sequenced at a high depth of coverage to enable within-host viral variant identification. Experimental results were assessed in conjunction with all publicly available genome sequences from SARS2-infected felids for evidence of felid-specific adaptation. Our results demonstrate the rapidity of SARS2 adaptation within-felid hosts, and highlight the importance of studying susceptible animal populations in close contact with humans, and cross-species transmission as a mechanism for novel viral variant emergence.

Results

Sample collection and viral titer

Twenty-three cats from three experimental cohorts were experimentally exposed to SARS2 through direct intranasal inoculation or cat-to-cat contact transmission (Fig. 1). Cat-to-cat transmission was facilitated by cohousing cats inoculated 24 hours prior with naïve cats. Nasal and oral lavage samples were obtained 1- or 3-days post-exposure and viral titers were assessed in plaque-forming units per milliliter (pfu/mL) (Supplementary Table S1). Viral titers from nasal lavage ranged from 0 to 6.2 log pfu/mL. For all samples with 0 log pfu/mL recovered from nasal lavage, ≥ 1.6 log pfu/mL was detected in the oral lavage. There was a significant positive relationship between dose received and titer recovered via nasal lavage ($P = 0.023$, $R^2 = 0.24$).

Genome sequencing and variant calling

Full viral genome sequences were recovered from RNA isolated from twenty-three cat-derived nasal lavage samples and three cell culture-derived viral stock samples (P1, P2, and P3). Two technical replicates were sequenced for each sample. Median and mean depths of coverage of the SARS2 genome were 2500 \times and 4100 \times respectively (Supplementary Fig. S1). Genomes were analyzed for single nucleotide and structural variants (SNVs and SVs) present in both technical replicates and ≥ 3 per cent of genome sequences (Fig. 2). There was no significant relationship between mean or median sequencing coverage and the viral titer measured for an individual cat on the date of sampling (linear models; $P = 0.076$, $R^2 = 0.14$ and $P = 0.56$, $R^2 = 0.016$, respectively) or between the mean or median depth of coverage and the number of within-host variants in a sample (linear model; $P = 0.59$, $R^2 = 0.012$ and $P = 0.93$, $R^2 = 0.00035$, respectively).

Across the twenty-three cat-derived samples, 118 SNVs and SVs in coding regions of the SARS2 genome were observed at ≥ 3 per cent allele frequency in two technical replicates within individual hosts (see Materials and Methods for more detail). Eighteen of these variants were also detected in the P1, P2, and P3 viral stock samples. Therefore, 85 per cent of variants detected in cats ($N = 100$) were not detected in the inoculum stocks at ≥ 3 per cent frequency. Sequence data were also analyzed for variants present at as low as 0.1 per cent frequency (Supplementary Fig. S2) to enable identification of variants that were initially present in the inoculum at low frequencies. Of the 118 variants detected in cats at ≥ 3 per cent allele frequency, 66 (56 per cent) were not detectable in the stock samples above 0.1 per cent frequency. Five hundred and fifty-seven variants were identified in cat-derived samples at ≥ 0.1 per cent frequency. Four hundred and forty of these (79 per cent) were not observed in the cell culture-derived viral stock samples at ≥ 0.1 per cent frequency.

Variant characteristics

We analyzed 118 variants occurring at ≥ 3 per cent frequency for type, predicted effect, and position in the SARS2 genome (Fig. 2). One hundred of 118 variants (85 per cent) were SNVs, and eighteen (15 per cent) were SVs. The 100 SNVs and 18 SVs were observed 246 and 65 times throughout the dataset, respectively, indicating that some were detected in more than one animal. The majority of SNVs ($N = 70$, 70 per cent) were nonsynonymous. SVs included nine frameshift variants, six disruptive inframe deletions, one disruptive inframe insertion, and a single conservative inframe insertion and deletion, respectively. We observed thirty-three variants

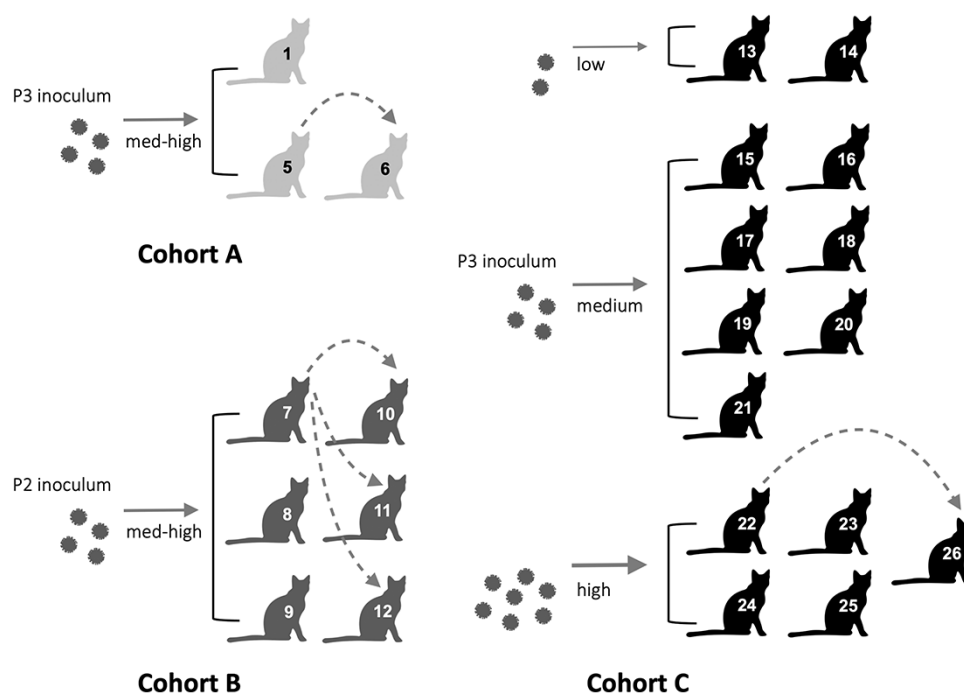


Figure 1. Twenty-three cats experimentally exposed to SARS2 were included in this study. Cohorts A and B were inoculated with a standard “medium-high” dose of 1×10^6 plaque-forming units (pfu). Cohort A included two directly inoculated cats and a contact cat; Cohort B included three directly inoculated and three contact animals; Cohort C included seven cats inoculated with a “medium dose” of 4.6×10^5 pfu; two cats inoculated with 1.0×10^4 pfu (“low” dose), and four cats inoculated with 2.1×10^7 pfu (“high” dose). One contact cat was exposed to the “high” dose animals in Cohort C. All contact cats were cohoused with directly inoculated cats one day post-infection. In Cohorts B and C, samples were collected from directly inoculated and contact cats three days post-exposure. In Cohort A, samples were collected from directly inoculated cats one day postinfection and from the contact cat six days post-exposure. Images from PhyloPic available under Public Domain Mark 1.0 license: Cat (<http://phylopic.org/image/23cd6aa4-9587-4a2e-8e26-de42885004c9/>) by David Orr and SARS-CoV-2 (<http://phylopic.org/image/81bc7804-4940-4fa5-a1ca-dd6c3a26aaa2/>) by Alissa Eckert and Dan Higgins.

in the spike, representing 26 per cent of all variants in a gene segment representing less than 13 per cent of the SARS2 genome. The next largest number of variants was in nonstructural protein 3 (nsp3). Nineteen variants (16 per cent) were detected in this segment which spans approximately 19 per cent of the genome.

Variants were further assessed for convergent emergence among cats, their relation to mutations present in human variant lineages, and the frequency of specific single nucleotide substitutions (Tables 1 and 2). Eleven of these variants were not detected at any level (>0.1 per cent allele frequency) in the viral stock inoculum, including three (D614G, S686G, and F59_Q76del) that are shared between two or more cats not connected by a transmission chain (Table 1). Fourteen variants originally derived from the viral stock inoculum were shared among three or more cats (Table 2). Eleven variants occur at the same genomic positions as mutations characteristic of human SARS2 lineages labeled as variants of concern by the World Health Organization and United States Centers for Disease Control and Prevention (CDC) (Tables 1 and 2). Eight are in the spike gene and occur at the same positions as mutations in Alpha, Beta, Gamma and Omicron variant lineages: H69R, F79L, D215H, D215N, D215_L216insKLRS, E484D, D614G, and H655Y; three others are in the nucleocapsid gene and share genomic positions with mutations in the Beta and Omicron variant lineages: P13S, G204_R209del, and T205I. We noted that C>T substitutions (C>U changes in the SARS2 genome) constituted the largest proportion (31 per cent) of single nucleotide changes observed in cats in this dataset (Supplementary Fig. S3).

Drivers of variant richness

The mean \pm SD number of SARS2 variants per cat was 12 ± 1.4 and the median was 10 ($N = 23$ samples; Fig. 3A). SARS2 genomes recovered from directly inoculated cats had significantly more variants than those from contact cats, which averaged 6.4 ± 0.4 variants per cat (Fig. 3B; t-test, $P = 0.00027$). There were also more variants in samples collected 3 dpi from directly inoculated cats than in samples collected 1 dpi (t-test, $P = 0.050$). There was no significant difference between the mean number of variants detected in cat-derived samples compared to cell culture-derived samples (t-test, $P = 0.59$). There was not a significant difference among the number of variants detected in directly inoculated cats belonging to the three infection cohorts (linear model; $P = 0.15$, $R^2 = 0.12$). There was also no significant difference between the number of variants in female and male cats or between juvenile and adult cats (t-tests, $P = 0.48$ and $P = 0.23$, respectively).

We also observed that the number of variants was higher in cats inoculated with higher doses of SARS2 (Fig. 3C), and a linear model of number of variants as a function of dose revealed a significant positive relationship between dose category and variant richness ($P = 0.022$, $R^2 = 0.38$). A linear model assessing log pfu/ml recovered from nasal lavage of directly inoculated cats as a function of both inoculation dose and dpi of sample collection was highly significant ($P = 0.0073$, $R^2 = 0.41$). However, there was no significant relationship between this measurement of viral titer and the number of viral variants detected in RNA extracted from the same samples (Supplementary Fig. S4, linear model, $P = 0.30$, $R^2 = 0.0079$).

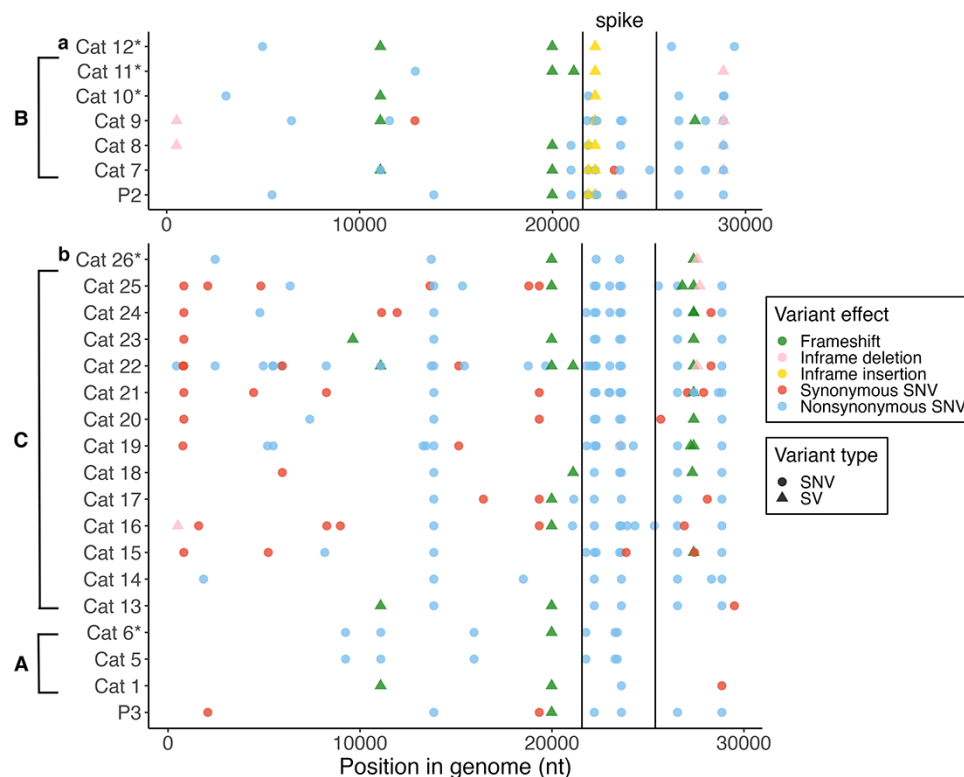


Figure 2. SARS2 within-host single nucleotide (SNV) and structural (SV) variants are detected at high frequency following infection of domestic cats. One hundred and eighteen variants were observed 311 times at $\geq 3\%$ allele frequency in 23 experimentally inoculated cats (median = 10 variants per cat). Cats were exposed to (a) Passage 2 (P2) or (b) Passage 3 (P3) cell culture-derived viral stocks. Each point represents a variant and point shape and color indicate variant type and predicted functional effect respectively. Cohorts are denoted on the left-hand side of the plot, and contact cats are indicated with an asterisk after their name along the y-axis. Cats 10, 11 and 12 were cohoused with Cats 7, 8 and 9. Cat 26 was cohoused with Cats 22, 23, 24 and 25. Cat 6 was cohoused with Cat 5. The location of the spike gene is delineated between two vertical lines.

Contact transmission of SARS2 infection and within-host variants

SARS2 was transmitted to five cats (Cats 6, 10, 11, 12, and 26) following contact with cats experimentally inoculated 1 day before cohousing. In Cohort A, Cat 6 was cohoused with one other cat, Cat 5, whereas in Cohorts B and C, contact cats were cohoused with multiple directly inoculated cats. Cats 10, 11, and 12 were cohoused with three other cats (Cats 7, 8, and 9). Cat 26 was cohoused with cats receiving a 'high' dose of SARS2 (Cats 22, 23, 24, and 25; [Supplementary Table S1](#), [Fig. 1](#)).

It was possible to deduce the infection donor for each contact cat by matching SARS2 variants detected in potential donors (at any level above 0.1 per cent allele frequency) with recipients ([Fig. 4](#)). In Cohort B, contact Cats 10, 11, and 12 each shared a larger proportion of within-host variants with Cat 7 (6/7, 4/5, and 3/6 variants, respectively) than with Cat 8 (4/7, 4/5, and 2/6, respectively) or Cat 9 (5/7, 3/5, and 2/6, respectively). In Cohort C, all seven variants in Cat 26 were also observed in Cat 22.

Putative donor Cats 7 and 22 also each had the highest viral titer assessed from nasal lavage of all directly inoculated cats in their cohorts, suggesting transmission was related to viral shedding competency. The viral titer recovered from contact cats varied ([Supplementary Table S1](#)). In some cases, the titer recovered from a contact cat was lower than that of the infection donor; in other cases, the contact cat had the higher titer.

Signatures of selection

We measured the nucleotide diversity, or the average number of pairwise changes at nonsynonymous and synonymous sites throughout the genome, to identify signatures of genomic selection. There was not a significant difference between nonsynonymous (π_N) and synonymous (π_S) nucleotide diversity (paired t-test, $P = 0.86$) across individual full viral genomes. However, signatures of selection emerged across our datasets at the gene level ([Fig. 5](#)). Notably, π_N was significantly greater than π_S in the spike gene, indicating positive or diversifying selection (paired t-test, $P = 0.00024$). In contrast, π_S was significantly greater than π_N in *orf1ab*, suggesting negative or purifying selection in this region (paired t-test, $P = 0.012$).

Global signatures of felid-specific adaptation of SARS2

One hundred and seventeen full-length SARS2 genomes isolated from domestic cats, and 128 genomes from other felid species were submitted to the GISAID EpiCoV public database between March 2020 and the end of March 2022. Ninety-three domestic cat and twenty-nine other felid sequences passed our selection criteria and quality control parameters and were used to generate time-measured phylogenies ([Fig. 6](#) and [Supplementary Fig. S5](#)). Custom Nextstrain builds were prepared separately for sequences from domestic cats alone ([Fig. 6](#)) and all felids ([Supplementary Fig. S5](#)). Relationships between SARS2 genomes did not appear

Table 1. Characteristics of eleven emergent variants detected in cats at high allele frequency (>50 per cent) or at genomic positions of interest, but not detected in the viral stock inoculum at any level (>0.1 per cent). Variants located at the same position as mutations in established SARS2 variant lineages are indicated with an asterisk. Variants detected in contact cats and representing part of a transmission chain from a directly inoculated cat are shown in the righthand column in italics.

Variant	Position in genome	Gene	Variant type and predicted effect	Host (allele frequency)
S348P	1847	nsp2	Nonsynonymous SNV	Cat 14 (56.1%)
L550F	2453	nsp2	Nonsynonymous SNV	Cat 22 (3.9%), Cat 26 (99.8%)
I750V	4967	nsp3	Nonsynonymous SNV	Cat 12 (90.1%)
D92A	13,715	nsp12	Nonsynonymous SNV	Cat 22 (3.7%), Cat 26 (99.8%)
L157F	18,508	nsp14	Nonsynonymous SNV	Cat 14 (95.7%)
D614G*	23,403	S	Nonsynonymous SNV	Cat 1 (0.7%), Cat 5 (98.9%), Cat 6 (98.3%), Cat 8 (1.9%)
S686G	23,618	S	Nonsynonymous SNV	Cat 1 (98.7%), Cat 21 (5.4%)
Y91Y	25,665	ORF3a	Synonymous	Cat 20 (72.6%)
F59_Q76del	27,567	ORF7a	Disruptive inframe deletion	Cat 22 (7.1%), Cat 26 (99.1%), Cat 25 (14.9%)
P13S*	28,310	N	Nonsynonymous SNV	Cat 14 (3.7%)
T205I*	28,887	N	Nonsynonymous SNV	Cat 7 (0.3%), Cat 10 (43.0%)

to be defined by the emergence of shared, felid-specific SNPs. Instead, sequences clustered based on date of sample collection and the predominant SARS2 human lineage at that time. Almost all sequences after spring 2020 carried the spike D614G mutation. Only one felid (a lion infected in April 2020) did not have this variant sequence. Although one snow leopard (infected in California in July 2021) appeared to diverge from the surrounding sequences, there was one matched sequence from an infected human in the same location and time period assigned to the same clade, suggesting that that particular felid sequence was still representative of some human infections in that area at that time.

Discussion

In response to the widespread, frequent infection of felids with SARS2, we investigated viral evolution and variant emergence in twenty-three cats from three experimental cohorts aimed at defining SARS2 infection characteristics in domestic cats. Cats were infected with SARS2 USA-WA1/2020, which represents one of the earliest culture-derived isolates (from a patient infected in January 2020) used in laboratory studies. We observed large numbers of within-host variants in viral genomes recovered from infected cats, with an average of twelve variants per cat sample. This stands in stark contrast to a reported mean of 1.4 variants per human sample (Lythgoe et al. 2021). This result is consistent with the rapid adaptive response of the virus to the cellular environment and immune defenses of a new host species in an experimental setting. Many variants observed in our study were

nonsynonymous, and predominated in the spike protein, indicating potential selection for improved binding and entry of feline host cells, host immune evasion, and other vital species-specific functions.

We witnessed the emergence of eleven SARS2 within-host variants identical to or within the same residue as mutations characteristic of known variant lineages that have emerged since SARS2 USA-WA1/2020 was isolated. The fact that these variants arose or were maintained after just one passage in cats signifies the strength of selective pressures in the feline hosts. Furthermore, it points to a potential mechanism for the introduction of sets of new SARS2 mutations into human populations. Spillover from an animal is a leading theory for the emergence of the Omicron variant lineage in December 2021 (Wei et al. 2021), notable for its large number of mutations, particularly in the spike, with the predominant alternative theory being evolution in an immunocompromised human (Li et al. 2022). Further, SARS2 spillover from mink, hamsters, and deer to people has been documented (Hammer et al. 2021; Munnink et al. 2021; Pickering et al. 2022; Yen et al. 2022), illustrating the potential for animal-to-human transmission of SARS2, even in animals that are not typically in proximity to humans. Our finding of an increase in SARS2 within-host variants in the viral spike after experimental infection of domestic cats highlights the potential for viral diversification and evolution, resulting in a SARS2 variant more adapted for spread in cats.

We also found that cat-derived variants can be transmitted through contact transmission from one cat to another, notwithstanding the potential random effects of bottlenecks and genetic drift on variant allele frequencies during natural transmission. Shared within-host variants allowed us to confirm the donor-recipient relationship despite multiple potential donors in Cohorts B and C and illustrated that contact cats tended to be infected by donor animals with a higher viral titer measured in nasal flush. The observed decrease in variant richness in contact cats is consistent with previous reporting of a narrow bottleneck during cat-to-cat transmission (Braun et al. 2021). The finding that higher number of variants occurred in animals exposed to higher viral titers supports the potential for variant emergence when viral exposure levels and viral shedding are high.

Although experimental infections cannot completely recapitulate natural infections and the stochastic processes that affect viral populations as they spread between and within hosts, they still provide valuable insights into the dynamics of viral evolution. They also provide the advantage of a controlled environment in which input and output viruses can be characterized. We used the same approach to sequence and call variants in samples of the viral inoculum and samples recovered from cats. This experimental design allowed us to identify what fraction of variants were present in low frequencies in the inoculum and increased in frequency in cats as compared to variants that were not detected in the inoculum and may have arisen *de novo*. There were consistently more of the latter group of putative *de novo* variants than low-frequency inoculum variants in our dataset, although proportions of each group varied as a function of the stringency of allele frequency cutoff used. Out of the 118 within-host variants present in cats at >3 per cent frequency, 52 (44 per cent) were detectable in viral stock samples and 66 (56 per cent) were not. It is also worth noting that the cell culture-derived viral stocks used to inoculate the cats contained variants that have been described previously as SARS2 adaptations to Vero cell culture (Bashor et al. 2021).

Table 2. Characteristics of fourteen emergent variants detected in both viral stocks and in cats. Emergent variants were selected based on allele frequency, genomic positions of interest and detection in multiple cats. Variants detected in at least one cat at high allele frequency (>50 per cent) but not detected at ≥ 3 per cent in viral stocks are indicated in bold. Variants located at the same position as mutations in established SARS2 variant lineages are indicated with an asterisk. All other variants were detected in three or more cats but not in the viral stocks at ≥ 3 per cent. Variant allele frequency measured in the viral stock inoculum (passaged three times in cell culture) is indicated in the righthand column.

Variant	Position in genome	Gene	Variant type and predicted effect	Number of cats ($>0.1\%$)	Inoculum (allele frequency)
V6V	823	nsp2	Synonymous SNV	8	P2 (0.5%)
L37F	11,083	nsp6	Nonsynonymous SNV	15	P1 (1.8%), P2 (1.8%)
T151fs	21,101	nsp16	Frameshift	9	P2 (1.9%)
H69R*	21,768	S	Nonsynonymous SNV	4	P1 (2.1%), P2 (1.2%)
F79L*	21,799	S	Nonsynonymous SNV	3	P1 (1.1%)
D215H*	22,205	S	Nonsynonymous SNV	16	P1 (4.2%), P2 (19.4%), P3 (9.7%)
D215N*	22,205	S	Nonsynonymous SNV	3	P1 (1.4%), P2 (1.5%)
D215_L216insKLRS*	22,206	S	Conservative inframe insertion	7	P2 (6.2%)
S247R	22,301	S	Nonsynonymous SNV	10	P2 (1.9%), P3 (0.8%)
E484D*	23,014	S	Nonsynonymous SNV	6	P3 (1.0%)
H655Y*	23,525	S	Nonsynonymous SNV	14	P1 (1.5%), P2 (4.8%), P3 (1.1%)
D61fs	27,381	ORF6	Frameshift variant and stop lost and splice region	12	P1 (0.2%), P2 (0.9%), P3 (0.7%)
S193_S194delinsT	28,850	N	Disruptive inframe deletion	7	P2 (2.5%)
G204_R209del*	28,878	N	Disruptive inframe deletion	5	P2 (0.3%)

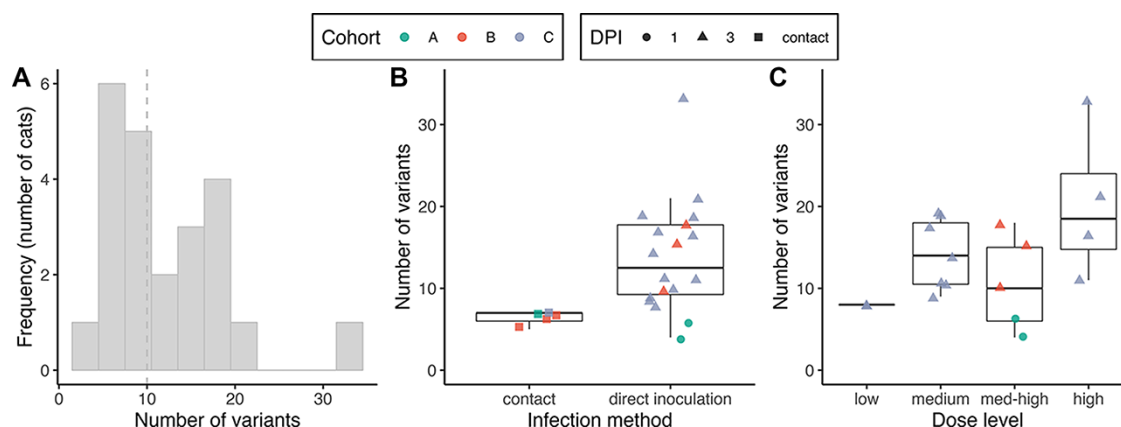


Figure 3. Method of infection and infection dose impact within-host variant detection. Variants detected in RNA recovered from SARS2-infected cats varied (A) among cats, (B) as a function of infection method and (C) by dose level. (A) Distribution of variant richness ranges from 4–33. Dashed line indicates the median (10). (B) More SARS2 variants were detected in directly inoculated cats ($N=18$) compared to contactinfected cats ($N=5$; t-test). (C) The mean number of variants was higher in samples from cats inoculated with higher doses of SARS2 virus ($N=18$ directly inoculated cats; linear model). In plots (B) and (C) each point represents an individual cat, and point shape and color indicate days post-infection (dpi) and infection Cohort respectively.

Cat-to-cat transmission findings demonstrate the general transmissibility of within-host variants and led us to question what might result from the onward transmission of cat-adapted SARS2 among cats. We analyzed publicly available SARS2 genomes from domestic cats ($N=93$) matched with SARS2 genomes recovered from temporally and geographically representative human patients ($N=543$). We found that naturally occurring feline SARS2 genomes were most closely associated with dominant SARS2 lineages present in human populations at

the time of sample collection. This is consistent with frequent transmission of SARS2 among humans and felids, although we were not able to trace individual spillover-spillback events with limited information about sample origin. Our analysis documents that felids have continued to be susceptible to newly emergent and increasingly transmissible human variant lineages over the course of the pandemic, as has been previously reported for several felid species (Shu and McCauley 2017; Curukoglu et al. 2021; Ferasin et al. 2021; Karikalan et al. 2021; Keller et al.

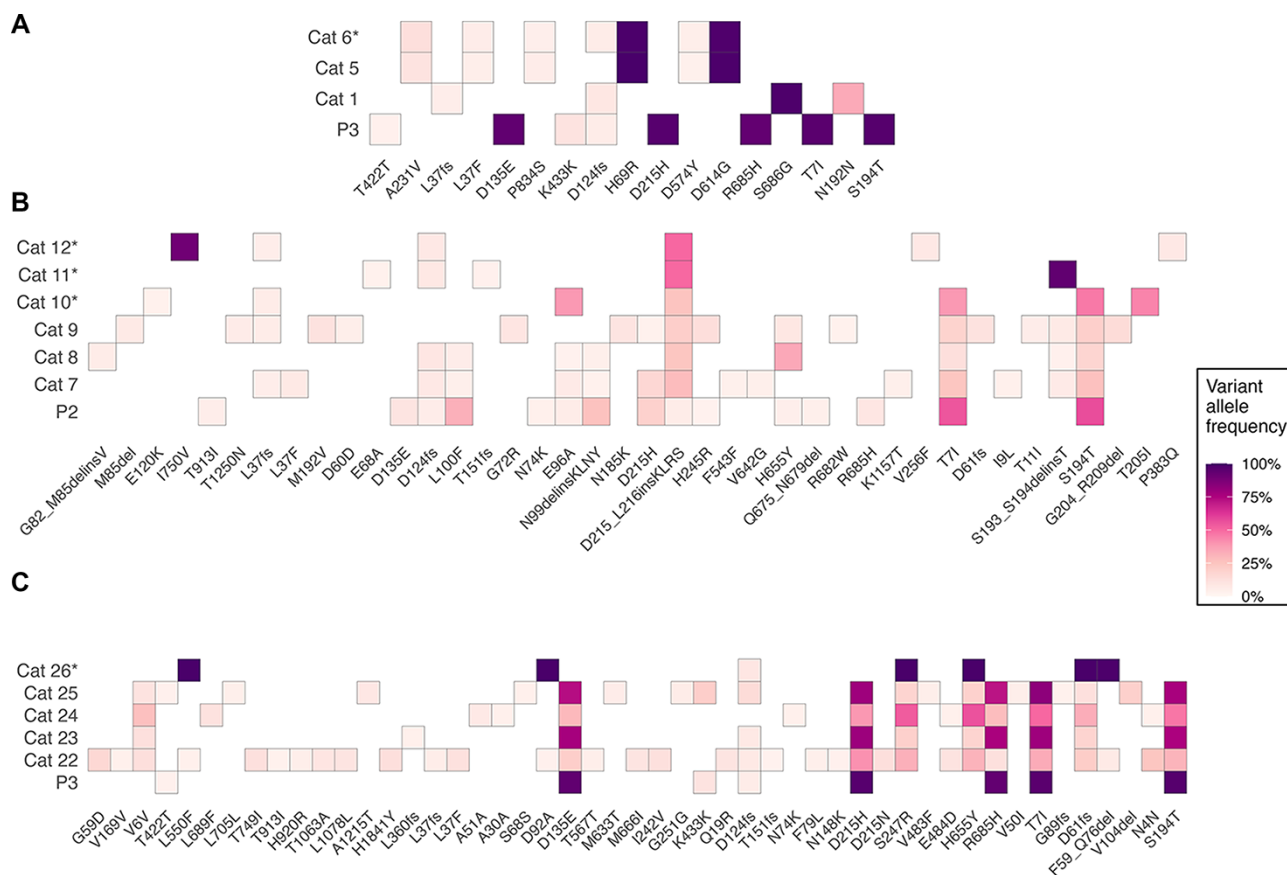


Figure 4. SARS2 within-host variants are transmitted between cats in three experimental cohorts. Each plot presents variants detected at $\geq 3\%$ allele frequency in contact cats, directly inoculated cats with which they were cohoused, and the viral stock used for inoculation. In plot (A) contact Cat 6 was infected by contact with Cat 5 which was inoculated with Passage 3 (P3) cell culture-derived viral stock. In plot (B) contact Cats 10, 11 and 12 were cohoused with Cats 7, 8 and 9 which were originally inoculated with Passage 2 (P2) viral stock. In plot (C), contact Cat 26 was cohoused with Cats 22, 23, 24 and 25, originally inoculated with P3 viral stock. Each tile represents a variant and color indicates variant allele frequency. Contact cats are denoted with an asterisk after their name along the y-axes. Code to prepare plots was modified from the R package for outbreak.info: (<https://github.com/outbreak-info/R-outbreak-info>).

2021; Mishra et al. 2021; Barroso-Arévalo et al. 2022; Zoccola et al. 2021).

Ultimately, our experimental infection system provided us with unique information that would be difficult or impossible to obtain in a natural setting. In particular, we were able to sequence both the input and output viruses, for both direct inoculations and contact transmissions, and we were able to assess viral titer at relevant timepoints. This allowed us to determine that a higher viral titer was more likely to result in cat-to-cat transmission when a naïve cat was cohoused with a group of infected cats. This is consistent with reported SARS2 transmission dynamics in humans (Goyal et al. 2021; Marks et al. 2021). A limitation of our approach was that we sequenced samples at a single timepoint, so we were unable to document changes in frequency of within-host variants over time. However, we observed shared variants transmitted from infection donor to recipient cats in five independent contact transmission events, affirming that our samples were collected at a biologically relevant timepoint.

It is widely accepted that SARS2 originally spilled over from an animal host (Wu et al. 2020; Zhou et al. 2020), and viral spillback from farmed mink (Hammer et al. 2021; Munnink et al. 2021), hamsters (Yen et al. 2022), and deer (Pickering et al. 2022) to humans has been reported. In our phylogenetic analysis of naturally occurring felid sequences, we were not able to discern specific consensus variants occurring in cats before widespread

circulation in people, and felid-to-human SARS2 transmission has not been confirmed by sequence analysis from intensive and coincident animal and human sampling. However, as evidenced by our and other analyses of reported SARS2 genomes, human-to-felid transmissions are frequent (Barrs et al. 2020; Patterson et al. 2020; Temmam et al. 2020; Zhang et al. 2020; Calvet et al. 2021; Fritz et al. 2021; Hamer et al. 2021; Villanueva-Saz et al. 2021). Of all animals with documented SARS2 susceptibility, humans are most likely to be in direct and intimate contact with the domestic cats living in 25 per cent of households in the United States (American Veterinary Medical Association 2017). Methods for documenting cat-to-human transmission have been described and will require intentional and continued research (Totton, Sargeant, and O'Connor 2021). Epidemiological and next-generation sequencing methods to assess cat-to-human transmissions are well within the capabilities of researchers globally, and the continued prevalence of SARS2 points to the urgency for ongoing monitoring of domestic cats and other animals in close contact with humans for evidence of SARS2 variant emergence and reservoir persistence.

Methods

Experimental exposures and sample collection

Directly inoculated cats in this study were exposed to viral stocks derived from an original SARS2 isolate obtained from the

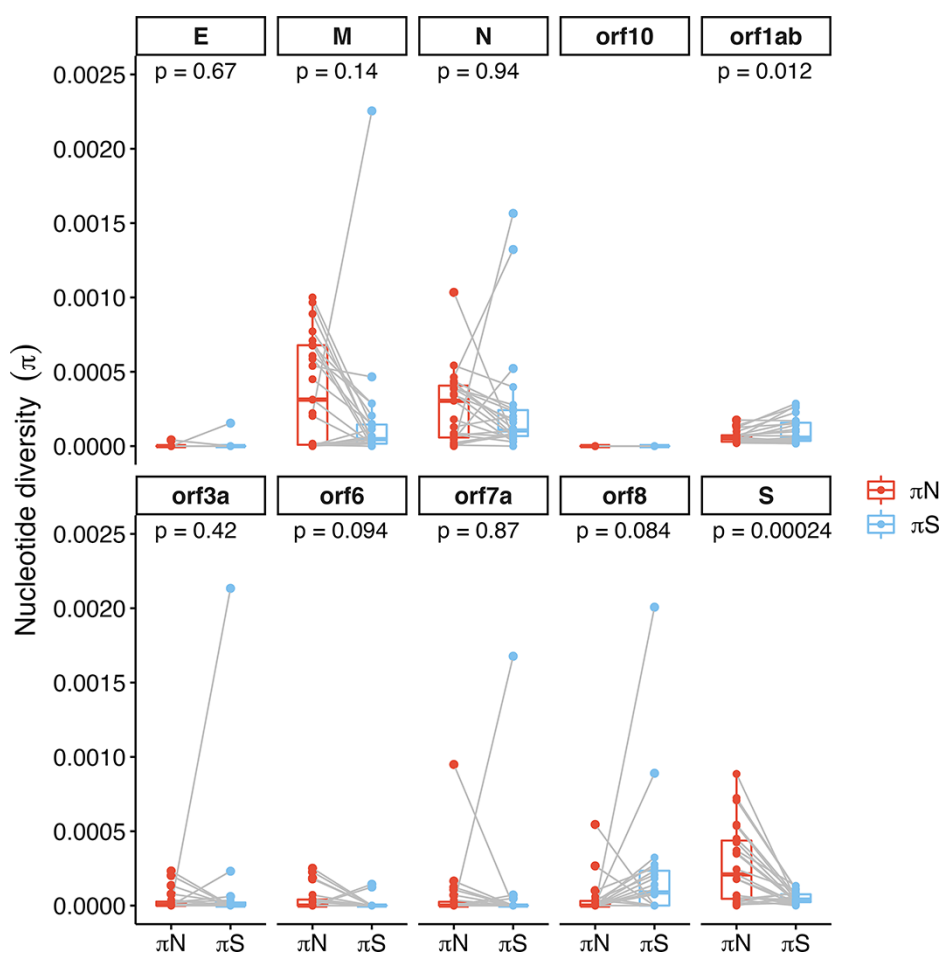


Figure 5. The SARS2 spike gene is under strong positive selection compared to other gene segments. Values of nonsynonymous (π N) and synonymous (π S) nucleotide diversity were calculated for each SARS2 gene or open reading frame across a dataset of 23 experimentally infected cats. Each point represents mean π N (red) or π S (blue) for two technical replicates from an individual cat in the indicated SARS2 gene region. P-values are indicated for paired t-tests between mean π N and mean π S.

Biodefense and Emerging Infections Research Resources Repository (<https://www.beiresources.org/>). The USA-WA1/2020 isolate (Genbank MN985325) was expanded in Vero cells over three passages (P1, P2, and P3) as previously described (Bashor et al. 2021). Three cohorts of adult (5–8 years) and juvenile (12 weeks) domestic cats ($N=23$) were intranasally inoculated with P2 or P3 virus stock in Animal Biosafety Level 3 facilities at Colorado State University. Cat cohorts are depicted in Fig. 1 and additional relevant metadata are provided in Supplementary Table S1.

Cohort A was part of a study to assess domestic cat susceptibility to SARS2 with previously reported results (Bosco-Lauth et al. 2020b). Cats 1 and 5 were inoculated with a ‘med-high’ dose of 1×10^6 pfu of P3 virus stock, and Cat 6 was cohoused with Cat 5 1 dpi to facilitate contact transmission. For both direct- and contact-exposed cats, dpi refers to the number of days following exposure of the directly inoculated cats in the cohort. Infection outcomes, including viral titer at 1 dpi (Cats 1 and 5) and 7 dpi (Cat 6), virus neutralization, seroconversion, and cat-to-cat transmission, were assessed as previously described (Bosco-Lauth et al. 2020b). Sequencing and within-host variant analysis for Cats 1, 5, and 6 were previously reported (Bashor et al. 2021), and data were reanalyzed in this study in light of the increased information available from two additional cohorts of experimentally exposed cats.

Cohort B was inoculated to assess age-related susceptibility to SARS2 (Bosco-Lauth, unpublished data). Cats 7–9 were inoculated with a ‘med-high’ dose of 1×10^6 pfu of P2 virus stock and were cohoused with three naïve cats (Cats 10–12) at 1 dpi to facilitate contact transmission. Viral titer at 3 dpi (Cats 7–9) and 4 dpi (Cats 10–12) was assessed for all six Cohort B cats. Cohort C was inoculated as part of a dose-response study SARS2 (Bosco-Lauth, unpublished data). Cats 13–25 were inoculated with three doses of P3 virus stock: low (Cats 13 and 14; 1.05×10^4 pfu), medium (Cats 15–21; 4.65×10^5 pfu), or high (Cats 22–25; 2.1×10^7 pfu). Cat 26 was cohoused with Cats 22–25 at 1 dpi to facilitate contact transmission. Viral titer was assessed at 3 dpi for Cats 13–25 and 4 dpi for Cat 26. Within-host variant analysis for Cohort B and C animals was conducted opportunistically following viral recovery in order to learn more about SARS2 adaptation in domestic cats.

In all studies, infections were performed as described previously (Bosco-Lauth et al. 2020b). Briefly, animals were lightly anesthetized before inoculation via the nares. Nasal lavage and oral swab samples were collected 1–3 days post-exposure. Nasal lavages were performed through the dropwise instillation of 1 mL BA-1 into the nares of awake or lightly anesthetized cats. Discharge was collected in a sterile petri dish. Oral swabs were obtained and stored in BA-1 medium (Tris-buffered Minimum

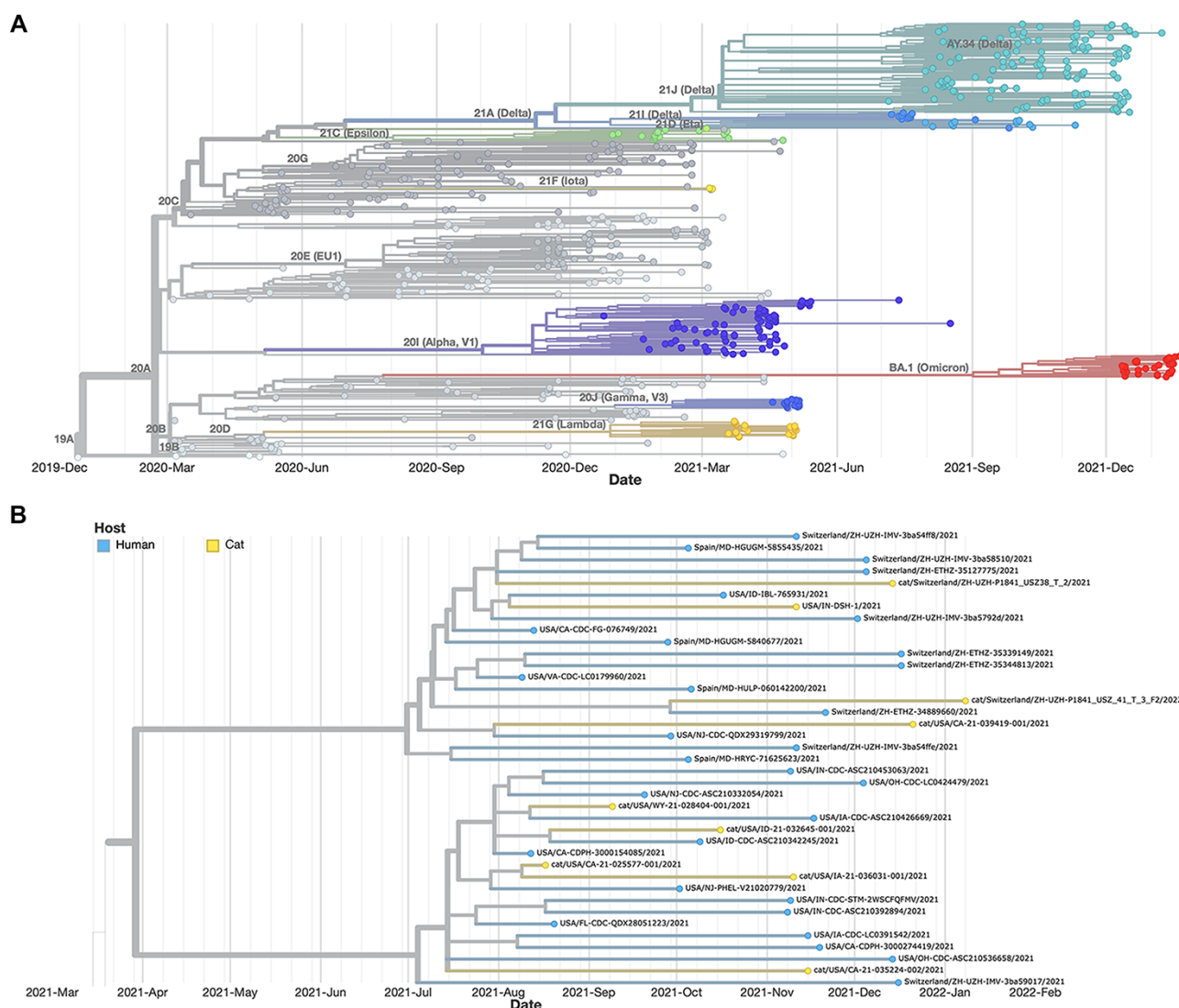


Figure 6. Domestic cat-derived SARS2 genomes reflect lineages of SARS2 circulating in humans during the same time period. SARS2 sequences recovered from (A) naturally infected domestic cats ($N=93$) and humans ($N= 543$) between March 2020 and March 2022. Sequences are colored by clade as assigned by Nextstrain. The interactive version of this phylogeny is hosted at: <https://nextstrain.org/community/laurbashor/SARS2felids/catshumans>. (B) A highlight from the same phylogeny showing a subset of sequences assigned to the Delta clade demonstrating the lack of species-specific patterns. Sequences are colored by host species (blue=human; yellow=cat). Data were obtained from the GISAID database on April 4, 2022, analyzed with the Augur pipeline and visualized with the Auspice web application.

Essential Medium with 1 per cent Bovine Serum Albumin) supplemented with gentamicin, amphotericin B, and penicillin/streptomycin. Viral titers (measured in pfu/mL) were assessed from nasal lavage samples from each individual.

RNA isolation and sequencing

Nasal lavage samples used to assess viral titer (collected at 1, 3, 4, or 7 dpi as indicated above) were then subjected to RNA extraction, complementary DNA (cDNA) generation and library preparation performed as previously described (Bashor et al. 2021). Briefly, nasal lavage fluid was inactivated in Trizol (100 μ L fluid in 900 μ L Trizol) and frozen at -80°C before RNA extraction. RNA was isolated using a modified Zymo RNA Clean and Concentrator 5 kit (Zymo Research) with DNase I (New England Biolabs). cDNA was generated with 10 μ L RNA and SuperScript II RT (Thermo Fisher Scientific) as previously described and frozen at -20°C before tiled amplicon enrichment and library preparation.

Samples were enriched for SARS2 genomic material using a PCR protocol designed by the ARTIC Network (ARTIC Network). This method employs a pooled primer scheme that generates ~ 400 bp overlapping ("tiled") amplicons that span the full viral genome. All samples were amplified in technical duplicates with ARTIC version 3 (V3) primers except for Cat 5 which was amplified singly with the previous ARTIC version (V2) primers. ARTIC primer sequences are publicly available here: https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019. PCR products were visualized on a 1 per cent agarose gel and quantified with a Qubit dsDNA Broad Range Assay kit (Thermo Fisher Scientific). Sequencing libraries were prepared with 540 ng input DNA per sample using a NEBNext Ultra II DNA Library Prep kit, and replicate samples were individually barcoded with NEBNext Multiplex Oligos for Illumina (New England Biolabs). Ampure XP beads were used for cleanup and size selection (Beckman Coulter), and a single 0.6 \times size selection was carried out following adapter ligation. Libraries were pooled for sequencing on an Illumina MiSeq

instrument (Illumina) with v2 500 cycle 2×250 bp kits. All libraries were sequenced over the course of three sequencing runs on the same MiSeq instrument at the Colorado State University Next Generation Sequencing Facility. Cat 5 in Cohort A and its technical replicate were both sequenced on the same run as part of a pilot project in May 2020. For the three viral stock inoculum Passages (P1, P2, and P3), all cats in Cohort C, and Cats 1 and 6 in Cohort A, one technical replicate was sequenced in a run in September 2020, and the second technical replicate was sequenced as part of a second run in October 2020. Both technical replicates for all six cats in Cohort B were sequenced on the October 2020 run.

Bioinformatics and variant calling

Raw sequencing data in FASTQ file format were analyzed with a custom Nextflow (Di Tommaso et al. 2017) bioinformatics pipeline designed to call SNVs and SVs in viral populations. The pipeline is publicly available on Github (Stenglein 2021). Sequencing reads were trimmed for quality and adapters with Cutadapt (Martin 2011) and then aligned to the USA-WA1/2020 reference sequence (GenBank MN985325). Data were pre-processed with GATK (van der Auwera et al. 2013) followed by LoFreq (Wilm et al. 2012) variant calling at a minimum depth of coverage of $40\times$ and a minimum allele frequency of 0.1 per cent (variants were later filtered to ≥ 3 per cent frequency for further analysis). Variants were annotated, and functional effects were predicted with SnpEff and SnpSift (Cingolani et al. 2012a, 2012b). Pipeline output was further analyzed and visualized in R statistical software (R Core Team 2021).

We took the additional approach of running iVar (Grubaugh et al. 2019), a bioinformatics pipeline designed for within-host viral variant calling from amplicon sequencing data to validate our pipeline. Any variants that were not also detected in iVar were filtered out unless they were identified to be an artifact of the 3 per cent allele frequency cutoff, which iVar applies before averaging technical duplicates and viral_variant_caller applies after. Eleven variants detected in cats were filtered out at this step.

Nonsynonymous and synonymous nucleotide diversity were calculated with the SNPGenie pipeline (Nelson and Hughes 2015). Nucleotide diversity (π) is calculated as the mean number of pairwise differences per nonsynonymous or synonymous site in the genome for a population of sequences. These estimates are weighted by allele frequencies. This analysis was done at both the population level (the population of full viral genomes within a host) and by gene product.

Phylogenetic analysis

Consensus genome sequences used for phylogenetic reconstruction of felid-derived SARS2 were obtained from GISAID (Shu and McCauley 2017). Sequence identifiers, metadata, and acknowledgments are listed in Supplementary Tables S2 and S3. All complete SARS2 genome sequences from host species belonging to the family Felidae available on GISAID as of 4 April 2022 were downloaded. Species included cat (*Felis catus*), tiger (*Panthera tigris*), lion (*Panthera leo*), snow leopard (*Panthera uncia*), cougar (*Puma concolor*), fishing cat (*Prionailurus viverrinus*), and leopard cat (*Prionailurus bengalensis*). Sequences that were incomplete, low coverage (>5 per cent Ns), or passage through cell culture were removed before analysis, which included the only cougar and fishing cat sequences. For each felid-derived sequence, a random sample of up to ten human-derived SARS2 genome sequences was also obtained from the full GISAID EpiCoV database. These human-derived sequences were subsampled in the same country and division

during a 2-week period surrounding the collection date of each felid sequence. Subsampling employed metadata available from GISAID and the Nextstrain (Hadfield et al. 2018) Augur filter subcommand. Maximum likelihood time-resolved phylogenies were reconstructed and visualized from these sequence data using open source Nextstrain bioinformatic tools. Briefly, sequences and metadata were input into the Augur pipeline, where they were filtered, aligned to the Wuhan-Hu-1 reference (Genbank NC_045512), and a tree was inferred with TreeTime. Results were visualized with Auspice software (Fig. 6, Supplementary Fig. S5). Publicly available interactive versions of these custom Nextstrain builds are hosted online. To query the presence and location of specific consensus variants throughout the phylogeny, we used the interactive tools provided by Nextstrain to filter the displayed data by genotype.

Data and code availability

All SARS2 raw next-generation sequencing data used in this study are publicly available in the NCBI SRA database under BioProject PRJNA704947 (previously published sequencing data for P1, P2, and P3 of the viral stock and Cats 1, 5, and 6) (Bashor et al. 2021) and BioProject PRJNA842572 (all remaining cats). The bioinformatics pipeline used to analyze the data is publicly available at https://github.com/stenglein-lab/viral_variant_caller. Additional data including complete variant summary tables output by the pipeline listing all within-host variants detected within samples from cats and the P1, P2, and P3 stocks used for inoculations, and R scripts for data processing, statistical analysis, and visualization are available at <https://github.com/laurabashor/SARS2felids>. Custom Nextstrain builds for publicly available sequence data from domestic cats and felids are hosted at: <https://nextstrain.org/community/laurabashor/SARS2felids/catshumans> and <https://nextstrain.org/community/laurabashor/SARS2felids/felidshumans>.

Supplementary data

Supplementary data are available at Virus Evolution online.

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