Coronavirus testing indicates transmission risk increases along wildlife supply chains for human consumption in Viet Nam, 2013-2014

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Abstract

Outbreaks of emerging coronaviruses in the past two decades and the current pandemic of a novel coronavirus (SARS-CoV-2) that emerged in China highlight the importance of this viral family as a zoonotic public health threat. To gain a better understanding of coronavirus presence and diversity in wildlife at wildlife-human interfaces in three southern provinces in Viet Nam 2013-2014, we used consensus Polymerase Chain Reactions to detect coronavirus sequences. In comparison to previous studies, we observed high proportions of positive samples among field rats (34.0%, 239/702) destined for human consumption and insectivorous bats in guano farms (74.8%, 234/313) adjacent to human dwellings. Most notably among field rats, the odds of coronavirus RNA detection significantly increased along the supply chain from field rats sold by traders (reference group; 20.7% positivity, 39/188) by a factor of 2.2 for field rats sold in large markets (32.0%, 116/363) and 10.0 for field rats sold and served in restaurants (55.6%, 84/151). Coronaviruses were detected in the majority of wildlife farms (60.7%, 17/28) and in the Malayan porcupines (6.0%, 20/331) and bamboo rats (6.3%, 6/96) that are farmed. We identified six known coronaviruses in bats and rodents, clustered in three *Coronaviridae* genera, including the *Alpha-, Beta-*, and *Gammacoronaviruses*. Our analysis also suggested either mixing of animal excreta in the environment or interspecies transmission of coronaviruses, as both bat and avian coronaviruses were detected in rodent feces in the trade. The mixing of multiple coronaviruses, and their apparent amplification along the wildlife supply chain into restaurants, suggests maximal risk for end consumers and likely underpins the mechanisms of zoonotic spillover to people.

Key words: coronavirus, One Health, rodents, bats, wildlife trade, wildlife farm, Viet Nam, surveillance, guano farming, amplification

Introduction

Human-wildlife contact with a bat or an intermediate host species in China likely triggered a coronavirus spillover event that may have involved wildlife markets and led to the pandemic spread of SARS-CoV-2 [1,2]. The pandemic risk of commercial trade in live wildlife was first recognized during the 2002-2003 Severe Acute Respiratory Syndrome (SARS) outbreak due to SARS-CoV [3]. This virus spread to more than 29 countries in Asia, Europe, and the Americas with 8,096 people infected and 774 deaths, costing the global economy about $US 40 billion in response and control measures [4,5]. Unfortunately, the global impact of COVID-19, the disease caused by SARS-CoV-2 has reached nearly every country and greatly surpassed those numbers by many orders of magnitude [6]. While bats are thought to be the ancestral hosts for all groups of coronaviruses, including those that were previously thought to be in the rodent and avian clades [7], for both SARS-CoV and SARS-CoV-2 wildlife trade supply chains are suspected to have contributed the additional conditions necessary for the emergence, spillover, and amplification of these viruses in humans [8,9]. To better understand the presence and diversity of coronaviruses in wildlife we conducted coronavirus surveillance at high-risk interfaces in Viet Nam from 2009 to 2014 [10]. We sampled in live field rat trade (*Rattus* sp. and *Bandicota* sp.) and wildlife farm interfaces to assess risk from different wildlife supply chains destined for human consumption, and sampled bat guano farms to assess the potential

occupational risk of this practice given that bat guano farm artificial roost structures are often erected near human dwellings.

In the early 2000s, the Vietnamese field rat trade was estimated to process 3,300-3,600 tons of live rats annually for consumption, a market valued at US$2 million [11]. Although rats are still commonly traded in wet markets and sold live for food consumption along the Mekong Delta in southern Viet Nam, no recent published data on the scale and scope of the trade is available [12]. This human-wildlife interface involves the capture of wild caught field rats, subsequent trade, and consumption along a supply chain involving the entire Mekong Delta region, particularly Cambodia and Viet Nam [13]. Driving this trade are consumers in Viet Nam and Cambodia, who report eating rats at least once per week because of their good flavor, low cost, and perception of rats as 'healthy, nutritious, natural, or disease free' [13]. Rodent parts (heads, tails, and internal organs discarded at slaughter) are also often fed to domestic livestock or herptiles raised in captivity including frogs, snakes, and crocodiles [12]. Records of this local trade in field rats include official rat hunts, instituted by French administrators, that killed upwards of 10,000 rats a day prior to the arrival of bubonic plague in Ha Noi in 1903 [14].

Over the past three decades, commercial wildlife farming has developed in many countries in Southeast Asia, including Viet Nam. Although there are historic references to the occurrence of wildlife farms in Viet Nam dating back to the late 1800s, the rapid expansion in terms of farm numbers, species diversity, and scale of operations has occurred in recent decades in response to growing domestic and international demand for wildlife [15]. A 2014 survey across 12 provinces in southern Viet Nam identified 6,006 registered wildlife farms of which 4,099 had active operations. The surveyed farms were stocked with approximately one million wild animals including, rodents, primates, civets, wild boar, Oriental rat-snakes, deer, crocodiles,

118 and softshell turtles. Ninety-five percent of the farms held 1-2 species of wildlife, and 70% of the
119 farms also raised domestic animals on the same premises [16]. A key component of the wildlife
120 farm industry in Viet Nam is the raising of wild species for meat for human consumption [16].
121 These farms sell to urban wild meat restaurants serving increasingly affluent populations
122 throughout the country and also supply international markets with wild meat [17]. Commercial
123 wildlife farming in Viet Nam is part of the expanded international trade of wildlife that has been
124 hypothesized to contribute to the cause of global epidemics, such as SARS [18] and now
125 COVID-19.

126 Emerging evidence suggests zoonotic virus spillover risk is a concern at bat-human
127 interfaces in Asia. Guano harvested from a cave in Thailand were positive for a group C
128 betacoronavirus, which includes MERS-CoV, and 2.7% of 218 people living in close proximity
129 to bats known to carry viruses related to SARS-CoV tested positive for SARS-related antibodies
130 in China [19,20]. The traditional practice of guano farming in parts of Cambodia and Viet Nam
131 involves the construction of artificial bat roosts in gardens or backyard farms, under which
132 domestic animals and crops are raised, and children often play [21,22]. Cambodian development
133 programs promoted the practice in 2004 to enhance soil fertility, reduce reliance on chemical
134 fertilizers, generate income ($US 0.50/kg), control insect pests, and protect the lesser Asiatic
135 yellow bats (*Scotophilus kuhlii*) that were being hunted [21–23]. No personal protection
136 measures are taken when harvesting the guano, which is used as fertilizer and is reported to
137 improve the growth rate in five economically important plant species [24].

138 In this study we investigated the presence and diversity of coronavirus sequences in the
139 field rat trade distribution chain, wildlife farms specializing in rodents for human consumption,

140 and bat guano "farms" and roosts near human dwellings to better understand the natural hosts of
141 coronaviruses and the risk for these interfaces to facilitate spillover into humans.
142

143 Materials and Methods

144 Sampling Locations

145 Sampling was performed at multiple sites representing several high-risk interfaces for
146 contacts among people, rodents, and bats. Rodent sampling focused on the live rodent trade
147 supply chain and rodent farms. Along the supply chain, we targeted eight sites involved in the
148 private sale and processing of live rodents for consumption, defined as 'traders' for the purpose
149 of this study in Dong Thap and Soc Trang provinces, 14 large markets sites in Dong Thap and
150 Soc Trang provinces (>20 vendors), and two restaurant sites in Soc Trang province (Fig 1). The
151 28 rodent farm sites we targeted in Dong Nai province produced Malayan porcupines (*Hystrix*
152 *brachyura*) and bamboo rats (*Rhizomys* sp.) for human consumption (Fig 2). Other species
153 observed or raised at the wildlife farm sites included dogs, cattle, pigs, chickens, ducks, pigeons,
154 geese, common pheasant, monitor lizards, wild boar, fish, python, crocodiles, deer, civets, non-
155 human primates as pets or part of private collections, free-flying wild birds, and free-ranging
156 peri-domestic rodents.
157

158 **Fig 1. Slaughtering rodents (left) and rodent market (right) in Dong Thap province,**
159 **October 2013**.
160

161 **Fig 2. Malayan porcupines (*Hystrix brachyura*) farm in Dong Nai province, November**
162 **2013.**
163

Bat sampling occurred at bat guano "farms" and a natural bat roost located at a religious site. Bat guano farms consisted of artificial roosts constructed with a concrete base and pillars topped with fronds of coconut palm or Asian Palmyra Palm (*Borassus flabellifer*) (Fig 3). Seventeen bat guano farms were sampled in the two provinces of Dong Thap and Soc Trang. The natural bat roost was located at a religious site in Soc Trang province known as the "bat pagoda", where *Pteropus* sp. have historically roosted in trees protected from hunting, and light and noise pollution [25].

Fig 3. Bat guano farms in Soc Trang Province, October 2013.

All study sampling occurred from January 2013 to March 2014 at 41 sites in the wet (south Viet Nam: May 1st - November 30th) and 30 in the dry (south Viet Nam: December 1st - April 30th) seasons. Given the distances between sites, all sites were sampled once except the bat pagoda natural roost in Soc Trang province, which was visited three times and sampled in both seasons.

Animal sampling

Samples were humanely collected using standard and previously published protocols [26]. Feces, swabs of the pen floors, and urine/urogenital swabs were collected from rodents at wildlife farms. Samples were classified as 'fecal sample' when collected from animals housed individually, and as 'environmental sample' when collected below cages housing multiple individuals. Samples from rodents in the trade included primarily oral swabs in addition to tissues (i.e. brain, kidney, lung, and small intestine), rectal swabs, and urine/urogenital swabs. These samples were collected from individual carcasses after the rodents were slaughtered by a

187 market vendor, trader, or restaurant kitchen staff. However, the rodents were usually butchered at
188 a common site for each observed time period that was only cleaned intermittently following the
189 trader's, vendor's, or restaurant's regular practices. Oral swabs were taken from the severed
190 heads, and additional tissue samples were collected from the internal organs and the
191 gastrointestinal tracts which were removed during the butchering process.

192 Fecal samples and a small number of urine samples from bats in guano farms and the
193 natural roost site were collected on clean plastic cover sheets within 1-2 hours after placement
194 under bat roosts, and thus each sample may represent one or multiple bats. Oral and rectal swabs
195 were also collected from live-captured bats at the natural pagoda roost site.

196 Animals were identified in the field to the lowest taxonomic level possible based on
197 morphological characteristics, and species was identified in a subset of animals through genetic
198 barcoding [15]. Due to difficulty of morphologic identification in the field, unless barcoded,
199 rodents (*Rattus argentiventer*, *R. tanezumi*, *R. norvegicus*, *R. exulans*, *R. losea,* and *Bandicota*
200 *indica*; [12,27]) were categorized as "field rats". Bats were classified as "*Microchiroptera*"
201 following the traditional taxonomic classification (new classification of two new suborders
202 *Yangochiroptera* and *Yinpterochiroptera*, was only published near the end of the study, so for
203 consistency we used the historical classification [28]).

204 All samples were collected in cryotubes containing RNAlater (RNA stabilization reagent,
205 Qiagen), and stored in liquid nitrogen in the field before being transported to the laboratory for
206 storage at -80 ˚C. Samples were tested by the Regional Animal Health Office No. 6 (RAHO6)
207 laboratory in Ho Chi Minh City. The study was approved by the Department of Animal Health of
208 the Ministry of Agriculture and Rural Development and protocols were reviewed by the

209 Institutional Animal Care and Use Committee at the University of California at Davis (protocol
210 number 16048).

211 Sample Testing

212 RNA was extracted (RNA MiniPrep Kit, Sigma-Aldrich) and cDNA transcribed
213 (SuperScript III First Strand cDNA Synthesis System, Invitrogen). Coronavirus RNA was
214 detected using two broadly reactive consensus nested-PCR assays targeting the *RNA dependent*
215 *RNA polymerase (RdRp)* gene [29,30]. The positive control was a synthetic plasmid containing
216 the primer-binding sites for both assays. Distilled water was used as a negative control and
217 included in each test batch. PCR products were visualized using 1.5% agarose gels, and bands of
218 the correct size were excised, cloned, and sequenced by Sanger dideoxy sequencing using the
219 same primers as for amplification.

220 Phylogenetic analysis

221 For sequence analysis and classification operating taxonomic units were defined with a
222 cut off of 90% identity, i.e. virus sequences that shared less than 90% identity to a known
223 sequence were labelled sequentially as PREDICT_CoV-1, -2, -3, etc. and groups sharing ≥ 90%
224 identity to a sequence already in GenBank were given the same name as the matching sequence
225 [7]. A phylogenetic tree was constructed for sequences amplified using the Watanabe protocol,
226 as this PCR protocol yielded longer sequences and more positive results than the Quan protocol.
227 Several representative sequences for each viral species found in our study were included for
228 analysis and are available in GenBank (Table S3). Alignments were performed using MUSCLE,
229 and trees were constructed using Maximum likelihood and the Tamura 3-parameter model in
230 MEGA7 [31]. The best-fit model of DNA substitution was selected in MEGA7 using BIC scores
231 (Bayesian Information Criterion) and Maximum Likelihood values (*lnL*). Bootstrap values were

232 calculated after 1000 replicates. In addition, a median-joining network was constructed using
233 Network 5.0.0.3 [32] to explore phylogenetic relationships among bat coronavirus 512/2005
234 sequences at the intraspecies level, as haplotype networks may better represent the relationships
235 among viral sequences with low sequence diversity compared with phylogenetic trees [33].

236 Statistical analyses

237 Visualization of sampling locations in provinces in Viet Nam, along with the distribution
238 by species and interface was constructed with the ggmap, ggplot2, and sp packages [34]. All
239 analyses were done using R version 3.5.0 or higher (R Development Core Team, Vienna,
240 Austria). Data (S1 Data) and code (S1 R Code) are available in the supplementary materials. The
241 effect of risk factors (season, sub-interface type) was examined and limited to interfaces for
242 which the distribution of samples across factors could support the analysis. These included
243 season for *Pteropus* bat samples collected in the bat pagoda natural roost and the effect of season
244 and sub-interface for samples collected in the rodent trade in southern Viet Nam. Given the low
245 sample size, the effect of season for *Pteropus* bats samples positive for coronaviruses was
246 assessed using a Fisher exact test. The effect of season (dry, wet, with dry season as reference
247 category) and sub-interface type (trader, large markets, restaurants, with trader as reference
248 category) in traded rodent samples positive for coronaviruses was assessed with a mixed effect
249 multivariable logistic regression, with sites as random effect (i.e. grouping variable) using the
250 lme4 R package [35]. A p-value of less than 0.05 was considered statistically significant. The
251 95% binomial confidence intervals for proportions were calculated using binom.test in R.
252 The comparison of the proportion of coronavirus positives in different sample types was
253 performed on positive individuals sampled in the rodent trade with multiple sample types

collected per individual. We then calculated the proportion of individuals positive for each sample type, as a proxy for the probability of detection by each sample type.

Results

Detection of coronavirus by animal taxa and interface

A total of 2,164 samples collected between January 2013 and March 2014 from rodents and bats were tested for coronaviruses (Table 1, S1 Table). Assuming that non-invasive samples from bats and farmed rodents represented unique distinct individuals, these samples came from 1,506 individuals, including 1,131 rodents and 375 bats from 70 sites sampled in Dong Thap, Soc Trang, and Dong Nai provinces in the southern region near the Mekong River Delta (Fig 4).

Fig 4. Map of sampling sites by province and multi-panel plots showing individual counts of animals sampled by province, taxa, and interface. The color of each bar represents the animal taxonomic group sampled in Dong Nai, Dong Thap, and Soc Trang provinces. *Sciuridae* and *Rattus argentiventer* were only sampled one time apiece from wildlife farms.

Out of 70 sites, coronavirus positives were detected at 58 including 100% (24/24) of live rodent trade sites, 60.7% (17/28) of rodent wildlife farm sites, 94.1% (16/17) of bat guano farm sites, and at the one natural pteropid bat roost. Wildlife farms were only sampled in Dong Nai province and the live rodent trade and bat interfaces were sampled in Dong Thap and Soc Trang provinces (Fig 4).

275 **Table 1: Summary of coronavirus positives by taxa and interface. Co-infection is defined as the detection of two different**
276 **coronavirus taxonomic units in an individual animal.**
277

Taxa group	Interface	Sub-interface	Taxa group	% site positive	% individual positive	Viral species	# of co-infected animals
Rodents	Rodent trade	Trader	Field rat[a]	100% (8/8)	20.7% (39/188)	Murine coronavirus (n=36), Longquan aa coronavirus (n=5)	2
		Large market	Field rat[a]	100% (14/14)	32.0% (116/363)	Murine coronavirus (n=103), Longquan aa coronavirus (n=31)	18
		Restaurant	Field rat[a]	100% (2/2)	55.6% (84/151)	Murine coronavirus (n=70), Longquan aa coronavirus (n=20)	6
	Wildlife farm		*Hystrix* sp.	47.8% (11/23)	6.0% (20/331)	Bat coronavirus 512/2005 (n=19), Infectious bronchitis virus (IBV) (n=1)	0
			Rhizomys sp.	45.5% (5/11)	6.3% (6/96)	Bat coronavirus 512/2005 (n=5), Infectious bronchitis virus (IBV) (n=1)	0
			Rattus sp.[b]	100% (1/1)	100% (1/1)	Bat coronavirus 512/2005 (n=1)	0
			Sciuridae sp.	0% (0/1)	0% (0/1)		
Bats	Human dwelling	Natural bat roost					
			Pteropus sp.	100% (1/1)	6.7% (4/60)	PREDICT_CoV-17 (n=3), PREDICT_CoV-35 (n=1)	0
			Cynopterus horsfieldii	0% (0/1)	0% (0/2)		
		Bat guano farm	*Microchiroptera*[c]	94.1% (16/17)	74.8% (234/313)	PREDICT_CoV-17 (n=1), PREDICT_CoV-35 (n=38), Bat coronavirus 512/2005 (n=216)	21[d]
				82.9% (58/70)	**33.5% (504/1506)**		**47**

278 [a] Field rat here refers to a mix of *Rattus* sp. and *Bandicota* sp.

279 [b] This environmental sample collected from a porcupine cage on a porcupine farm was barcoded as *Rattus* sp., suggesting this species
280 was free-ranging at the site (Fig 2). The detection of a bat virus from this sample is suggestive of either environmental mixing or viral
281 sharing.
282 [c] Suborder
283 [d] Co-infections included PREDICT_CoV-17 with Bat coronavirus 512/2005 (n=1) and PREDICT_CoV-35 with Bat coronavirus
284 512/2005 (n=20).

285 Coronaviruses were detected in the field rat trade (a mix of *Rattus* and *Bandicota* genera)
286 at all sites in Dong Thap (n=16) and Soc Trang (n=8) provinces, with 34.6% (95% CI 29.8 –
287 39.7%, 129/373) and 33.4% (95% CI 28.4 – 38.9%, 110/329) positives respectively. The overall
288 proportion of positives in field rats was 34.0% (95% CI 30.6 – 37.7%, 239/702), ranging from
289 3.2% to 74.4% across sites. Field rats sampled in the rodent trade had an increasing proportion of
290 positives along the distribution chain. Starting with traders, the proportion positive was 20.7%
291 (95% CI 15.3 – 27.4%, 39/188), 32.0% (95% CI 27.2 – 37.1%, 116/363) in large markets, and
292 55.6% (95% CI 47.3 – 63.6%, 84/151) at restaurants (Fig 5). The proportion of positives was
293 higher in the wet season (36.7%, 95% CI 32.8 – 40.8%, 210/572) than the dry season (22.3%,
294 95% CI 15.7 – 30.6%, 29/130). In a multivariate model with site as random effect, both season
295 and interface type were significantly associated with the risk of rodent infection, with higher risk
296 of infection in the wet season (OR=4.9, 95% CI 1.4 – 18.0), and increasing risk along the supply
297 chain from traders (baseline) to large markets (OR=2.2, 95% CI 1.05 – 4.7), to restaurants
298 (OR=10.0, 95% CI 2.7 – 39.5) (S2 Table). It should be noted, however, that since sites were only
299 visited during one season, both independent variables were defined at the site level and
300 confounding effects with other site-level characteristics cannot be excluded.
301
302 **Fig 5. Plot of the proportion of coronavirus positives in field rats by interface. Bars show**
303 **95% confidence intervals.**
304
305 Among the positive field rats with more than one sample tested (n=220), the proportion
306 positive by sample type was 79.9% (95% CI 73.9 – 84.9%, 175/219) in oral swabs, 52.9% (95%
307 CI 38.6 – 66.8%, 27/51) in lung, 51.6% (95% CI 43.5 – 59.7%, 80/155) in small intestine, 31.2%

308 (95% CI 12.1 – 58.5%, 5/16) in brain, 23.1% (95% CI 6.2 – 54.0%, 3/13) in kidney, 50.0% in
309 feces (1/2), 100% in spleen (1/1), and 0% in urine/urogenital swabs (0/1).
310 At the rodent farm interface, 6.0% (95% CI 3.8 – 9.3%, 20/331) of *Hystrix brachyura* and
311 6.3% (95% CI 2.6 – 13.6%, 6/96) of *Rhizomys* sp. were positive. The overall proportion of
312 positives was 6.3% (95% CI 4.3 – 9.1%, 27/429) (Table 1 and Fig 4). There was no difference
313 among species or season and proportion positive in rodent farms, and low sample size and
314 unequal sampling limited analysis.
315 The proportion of coronavirus positives at the two bat interfaces differed by an order of
316 magnitude as 74.8% (95% CI 69.5 – 79.4%) of the non-invasive samples collected from
317 *Microchiroptera* bats at bat guano farms were positive, and 6.7% (95% CI 2.2 – 17.0%) of the
318 *Pteropus* genus samples at the natural roost in Soc Trang province (Fig 4) were positive (Table
319 1). Pteropid bats sampled at the natural roost had higher proportions of positives in the wet
320 season (27.3%, 95% CI 7.3 – 60.7%, 3/11) compared with the dry season (2.0%, 95% CI 0.1 –
321 12.2%, 1/50; Fisher exact test p=0.02, OR=16.6 [1.2 – 956.8]), although low sample size and
322 single sampling per season warrants cautious interpretation.
323

324 Phylogenetic analysis

325 Six distinct taxonomic units of coronaviruses corresponding to bat coronavirus 512/2005,
326 Longquan aa coronavirus, avian infectious bronchitis virus (IBV), murine coronavirus,
327 PREDICT_CoV-17, and PREDICT_CoV-35 were detected. All these viruses were detected
328 using both the Watanabe and Quan assays, except IBV sequences that were detected only using
329 the Quan protocol. Of the 504 positive animals, 433 were positive by the Watanabe assay, 410
330 were positive by the Quan assay, and 339 were positive by both. Phylogenetic analysis showed

331 that among the six coronaviruses detected, PREDICT_CoV-35 and bat CoV 512/2005 clustered
332 within the *Alphacoronaviruses*, while PREDICT_CoV-17, Longquan aa CoV and murine CoV
333 clustered within the *Betacoronaviruses*. The virus identified within the *Gammacoronavirus*
334 genus was avian IBV.

335 PREDICT_CoV-17 and PREDICT_CoV-35 were first reported by Anthony et al. [17].
336 We found PREDICT_CoV-17 in *Pteropus* bats and in *Microchiroptera* (Table 1). The
337 PREDICT_CoV-17 sequences from *Pteropus* detected in this study clustered closely with
338 PREDICT_CoV-17 sequences from *Pteropus giganteus* bats in Nepal and *Pteropus lylei* bats in
339 Thailand [36] (Fig 6, S3 Table). PREDICT_CoV-35 was found in *Microchiroptera* in bat guano
340 farms and in a pteropid bat (Table 1). PREDICT_CoV-35 sequences from Viet Nam clustered
341 with other PREDICT_CoV-35 sequences found previously in samples from hunted *Scotophilus*
342 *kuhlii* bats in Cambodia (S3 Table; Dr. Lucy Keatts personal communication), and with
343 sequences found in bats from an earlier study in the Mekong Delta region in Viet Nam (Fig 6).

344 Bat coronavirus 512/2005 was detected in *Microchiroptera* bat guano; and in *H.*
345 *brachyura* (feces and environmental samples), *R. pruinosus* (feces barcoded), and *R.*
346 *argentiventer* (barcoded environmental sample) in wildlife farms (Table 1 and S1 Table). In
347 *Microchiroptera*, Bat coronavirus 512/2005 was frequently found in co-infection with
348 PREDICT_CoV-35 (Table 1, S1 Table). Network analysis showed the relationships among the
349 bat coronavirus 512/2005 sequences from the three provinces in south Viet Nam (Fig 7). We
350 observed two main clusters and a shallow geographic structure of genetic diversity, perhaps
351 illustrative of sampling effort but also of localized transmission and circulation of bat
352 coronavirus 512/2005 strains in these provinces. One cluster was exclusively detected in
353 *Microchiroptera* and mostly restricted to Dong Thap province and another cluster included

354 sequences shared among all hosts and distributed in the three provinces (Fig 7). Parts of the
355 network showed a star-like topology (Fig 7), typical of populations in expansion that have
356 recently increased size. There were three sequence types that were shared among
357 *Microchiroptera* and rodents.

358 Murine coronavirus and Longquan aa coronavirus were detected in 209 and 56 field rat
359 samples, respectively, and 26 were coinfected with both (Table 1). Two sequences of IBV were
360 detected in rodent feces collected on two wildlife farms, one in a bamboo rat and another in a
361 Malayan porcupine. The rodent interfaces where bat and avian coronaviruses were detected in
362 feces were not full containment facilities and possibly had bats and birds flying and roosting
363 overhead (Fig 2). The IBV positives were detected in fecal samples from wildlife farms that had
364 chickens, pigs, and dogs on site.

365

366 **Fig 6. Phylogenetic tree of bat and rodent coronavirus sequences detected in Viet Nam**. The
367 analysis is based on 387 bp fragment of the *RdRp* gene using maximum likelihood with the
368 Tamura 3-parameter model, Gamma distributed with Invariant sites (G+I), and 1000 bootstrap
369 replicates via MEGA7. The analysis included 17 sequences from this study (red from bat hosts,
370 blue from rodent hosts), six sequences (in gray) from a previous study in Viet Nam [27], and 25
371 reference sequences (in black) available in the GenBank database (S3 Table). The tree was
372 rooted by a strain of Night-heron coronavirus HKU19 (GenBank accession No. NC_016994).

373

374 **Fig 7. Median-joining networks of bat coronavirus 512/2005 *RdRp* sequences color-coded**
375 **according to (A) host and (B) sampling location**. Each circle represents a sequence, and circle
376 size is proportional to the number of animals sharing a sequence. Numbers on branches indicate
377 the number of mutations between sequences (if >1). Circles are colored-coded by animal host:
378 bat (*Microchiroptera*), rodent (*Rattus* & *Bandicota*, *Rhizomys*, and *Hystrix*) and sampling
379 location (Dong Thap (blue), Dong Nai (yellow) and Soc Trang (green)). Small black circles
380 represent median vectors (ancestral or unsampled intermediate sequence types).

Discussion

High prevalence and amplification along the supply chain for human consumption

Significant findings of this study are the high proportion of coronavirus positive animals and the increasing proportion of positives found along the rodent trade supply chain from the capture site to restaurants. The transit of multiple animal species through the supply chain offers opportunities for inter- and intra-species mixing. Overcrowding and close confinement of live animals in cages results in increased animal contact, likely leading to stress. While methodologically similar to rodent surveys in Zhejiang province, China (2%), Dong Thap province, Viet Nam (4.4%), and globally (0.32%), our overall proportion of coronavirus positives was much higher among field rats (34.5%) and somewhat higher among farmed rodents (6.3%) [7,27,37]. Stress and poor nutrition likely contributes to shedding by reducing animal condition and altering immune functions [38]. Together, these factors may result in increased shedding and amplification of coronaviruses along the supply chain for human consumption.

The amplification of coronavirus along the supply chain may be associated with season as field rats were significantly more positive in the wet season. *Rattus argentiventer* generally reproduce year-round in Viet Nam, but are particularly abundant in the wet season (May through October) following the rice harvest when an abundance of food supports the population increase [39]. If these seasonal population increases affect density dependent contact, there could be increased coronavirus prevalence and shedding in wild field rats during certain times of the year, which could then be further amplified along the trade.

403 Our survey was not a comprehensive multi-year evaluation of the field rat supply chain
404 and it was restricted to two provinces with this interface. These limitations mean we are not able
405 to make inferences about larger spatial patterns or the inter-annual variability of coronavirus
406 prevalence in wildlife populations found in this interface, which spans into neighboring
407 Cambodia.

408 However, from a mechanistic perspective as animals progress along the wildlife supply
409 chain, opportunity for human contact increases, including close direct contact with traders,
410 butchers, cooks, and consumers [40]. The combination of increased coronavirus prevalence in
411 traded wildlife and greater opportunity for human-wildlife contact as well as intra- and inter-
412 species contact in trade systems is likely to increase the risk of zoonotic transmission of
413 coronaviruses in wildlife markets, restaurants, and other trade interfaces.

414 Viral sharing or environmental mixing

415 We detected avian and bat coronaviruses in wildlife farm rodents, including Malayan
416 porcupines and bamboo rats, but we did not detect rodent-associated coronaviruses. The only
417 previously published coronavirus testing of Malayan porcupine samples carried out in China
418 were negative [41]. It is unclear if the Malayan porcupine samples from animals screened in this
419 study were infected with the avian or bat viruses or if environmental contamination or mixing
420 occurred with avian and bat guano. Chickens were present at the two sites where the IBV-
421 positive rodents were detected, and bats fly and potentially roost overhead at most farms.
422 'Artificial market' studies of influenza A viruses have found cage-stacking of species on top of
423 other species and shared water sources facilitate viral transmission [42,43]. Nevertheless, viral
424 sharing between species and environmental contamination or mixing (i.e. bat/bird guano landing

425 on rat feces) are two equally likely explanations for the presence of bat and avian coronaviruses
426 detected in rodent fecal and environmental samples.

427 The field rats were co-infected with the Longquan aa coronavirus and the murine
428 coronaviruses, both of which are from the Lineage A (*Embecovirus*) *Betacoronavirus* genus. Co-
429 infections with multiple coronaviruses deserve particular attention as this co-occurrence may
430 facilitate viral recombination leading to the emergence of new viruses [44,45].

431 At the very least, we conclude that rodents in the field and farmed rodent supply chains
432 are being exposed to coronaviruses from rodents, bats, and birds and perhaps creating
433 opportunities for coronavirus recombination events, which may lead to viruses that could spill
434 over into humans [46]. Repeated and more direct individual sampling of these species at these
435 interfaces would be useful to determine if viral sharing was occurring versus environmental
436 contamination of samples.

437 Bat guano farms

438 The high proportion of positive bat feces at bat guano farms indicates the potential risk of
439 bat guano farmers, their families, and their animals being exposed to bat coronaviruses. The
440 overall proportion of positives (74.8%) was higher than previous studies using similar testing
441 methods targeting bats in Viet Nam (22%), Thailand (7.6%), Lao PDR (6.5%), and Cambodia
442 (4.85%) [27,47,48]. In this region of Viet Nam, artificial roosts are typically erected in backyard
443 family owned plots that incorporate a mosaic of duck, goat, or pig production and crops such as
444 guava tress or other fruit trees and large scale kitchen gardens.

445 Bats have been shown to be an important evolutionary hosts of coronaviruses, including
446 those infecting humans [7,49–52]. Both PREDICT_CoV-17 and PREDICT_CoV-35 have been
447 detected previously in the *Pteropus* and *Microchiroptera* bats in Viet Nam, Cambodia, and

448 Nepal, which confirms that coronaviruses are capable of infecting distantly related hosts [7]. The
449 finding of the same virus in different bat species raises the question of whether they co-roost
450 and/or share viruses through contact during other activities. Utilizing shared resources such as
451 water or feeding on and around crops and fruit could lead to contact and facilitate a host jump.
452 The presence of the same virus in bat species in multiple neighboring countries supports the
453 suggestion by others that virus distribution coincides with their bat host distribution [7,53,54].
454 While there has been no testing of the pathogenicity of these bat coronaviruses in humans or
455 animals, they are found at close contact bat-human interfaces and further characterization is
456 needed to understand their host range and potential for spillover. Any general persecution of bats
457 because of zoonotic viruses they may carry can actually increase the number of susceptible bats
458 and increase transmission risk to people [56], and would interfere with the important ecosystem
459 services that bats provide, such as controlling insect pests of rice fields [55], plant pollination,
460 and seed dispersal.
461

462 ## Capacity building and outreach

463 Beyond the viral findings, this work represented an important opportunity for capacity
464 development in field, laboratory, and scientific disciplines, as well as opportunities for social
465 engagement and education of high-risk communities on zoonotic disease threats. The consensus
466 PCR approach for viral detection provides a cost-effective tool to detect emerging viruses in low-
467 resource settings. Our work adds to the growing body of research demonstrating the utility of this
468 approach to detect both known and novel viruses and co-infections in a variety of taxa, sample
469 types, and interfaces. In Viet Nam, the direct result is an enhanced One Health surveillance
470 capacity to detect important emerging or unknown viruses in humans, wildlife, and livestock. In

471 the communities with which we partnered, strong engagement enabled teams to sample a wide
472 diversity of wild animals at high-risk interfaces. Importantly, we have returned to these same
473 communities to share the viral findings and to educate participants with an outreach program on
474 how to live safely with bats [57].

475

476 # Conclusions

477 Large percentages of coronaviruses were detected at high risk interfaces in bats and
478 rodents, which is of concern when assessing the potential for human exposure and spillover. The
479 observed viral amplification along the wildlife trade supply chain for human consumption likely
480 resulted from the mixing and close confinement of stressed live animals, such as field rats, and
481 sheds light on the potential for coronavirus shedding in other wildlife supply chains (e.g., civets,
482 pangolins) where similarly large numbers of animals are collected, transported, and confined.
483 Livestock and people living in close contact with rodents, bats, and birds shedding coronaviruses
484 provides opportunities for intra- and inter-species transmission and potential recombination of
485 coronaviruses.

486 Human behavior is facilitating the spillover of viruses, such as coronavirus, from animals
487 to people. The wildlife trade supply chain from the field to the restaurant provides multiple
488 opportunities for such spillover events to occur [1]. To minimize the public health risks of viral
489 disease emergence from wildlife and to safeguard livestock-based production systems, we
490 recommend precautionary measures that restrict the killing, commercial breeding, transport,
491 buying, selling, storage, processing and consuming of wild animals. The emergence of SARS-
492 CoV, MERS-CoV, and now SARS-CoV-2 highlight the importance of the coronavirus viral

493 family to affect global public health. The world must increase vigilance through building and
494 improving detection capacity; actively conducting surveillance to detect and characterize
495 coronaviruses in humans, wildlife, and livestock; and to inform human behaviors in order to
496 reduce zoonotic viral transmission to humans.
497

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513

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682

Supporting information

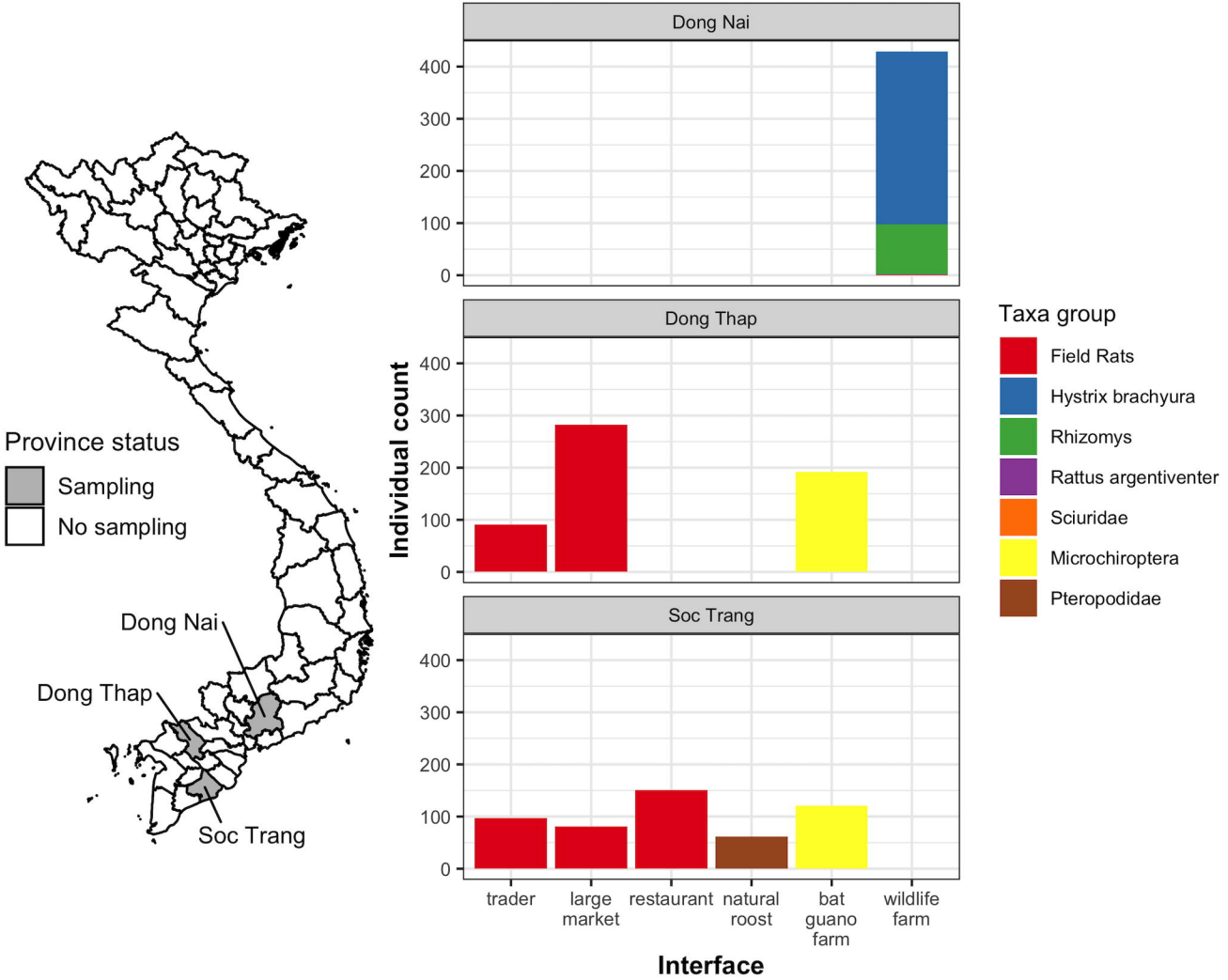
S1 Table. Summary of all testing results by genus, interface, sub-interface, sample types, sites, percentage of samples testing positive, and viral species.

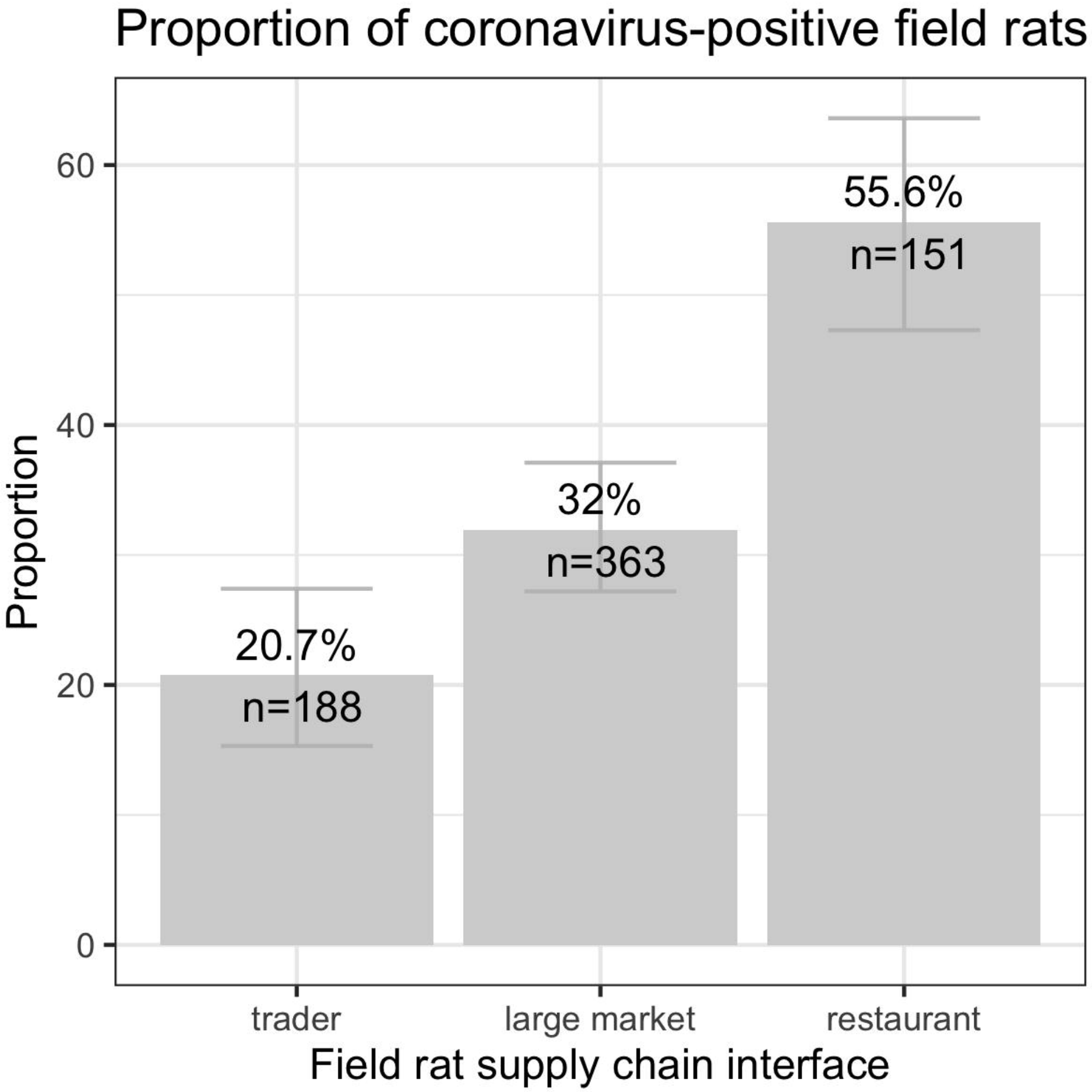
S2 Table: Multivariate mixed effect logistic regression showing the association between season and interface with coronavirus positives in field rats in the rodent trade.

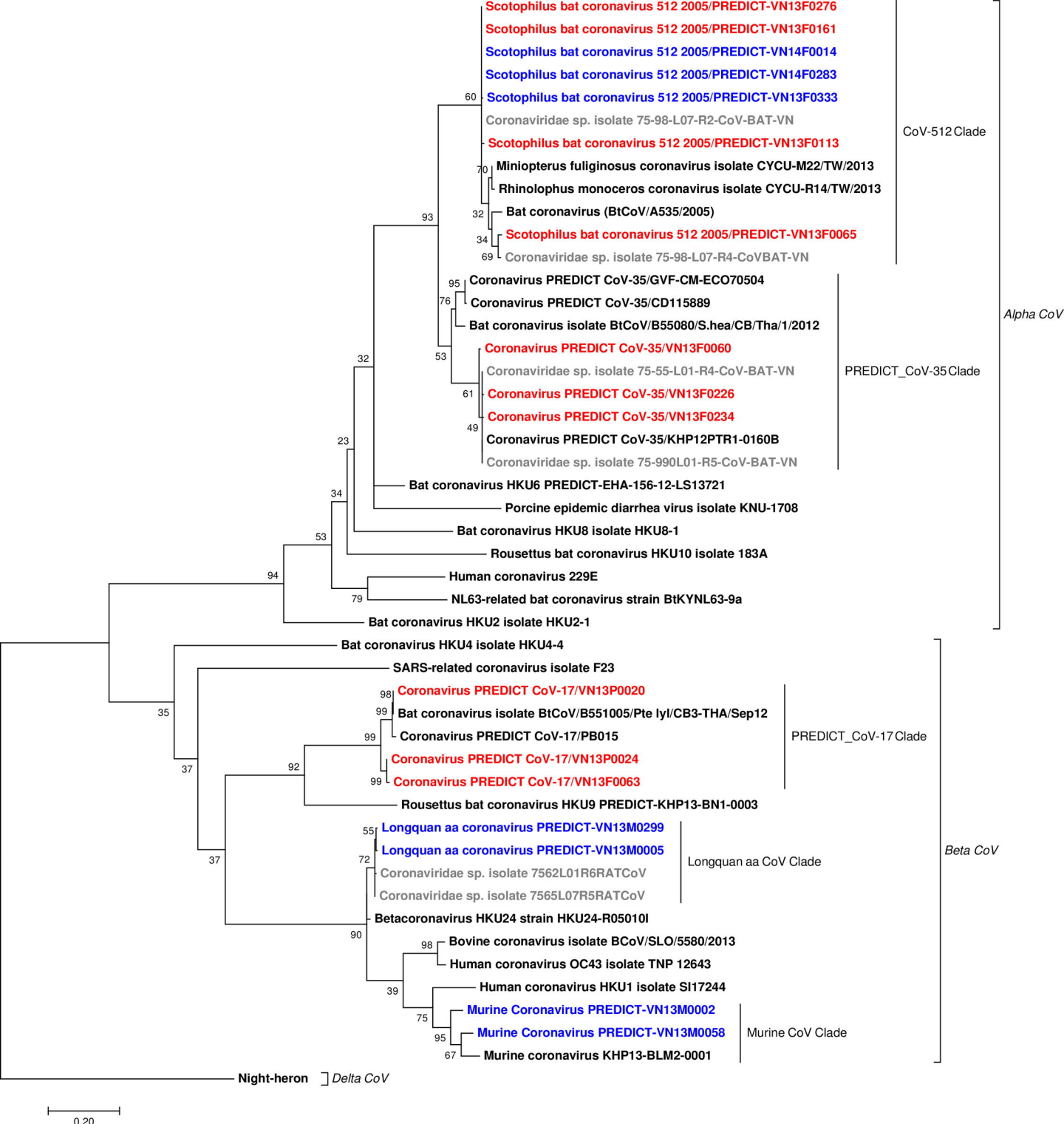
S3 Table: GenBank accession numbers for coronavirus sequences detected in this study and for reference sequences

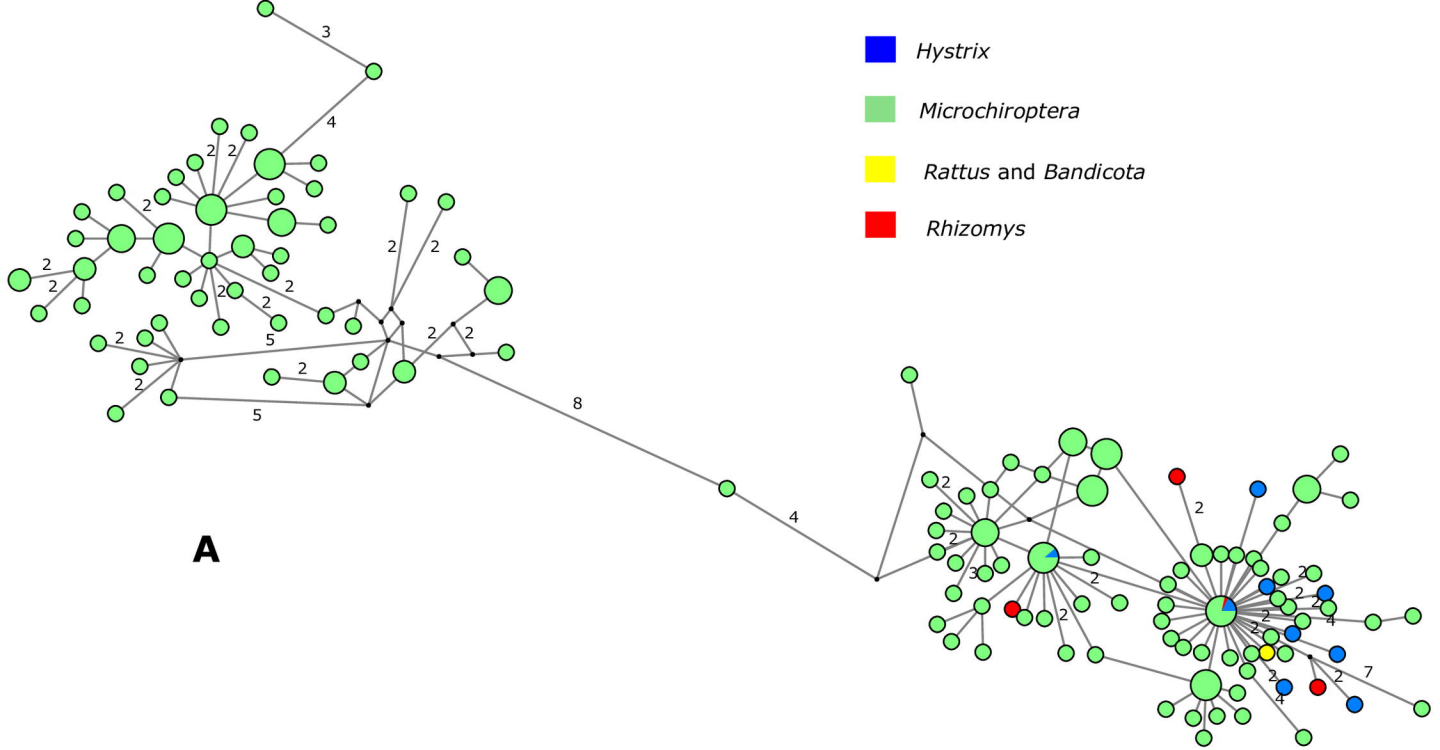
S1 Data. Data required for all analysis and metadata for each parameter is available at (pending DOI processing): https://doi.org/10.5061/dryad.7h44j0zrj OR https://datadryad.org/stash/share/pk3wVUxFNzTuCYZ9t8haKRPmx7V8YhTDBuHpG8JJ9kU

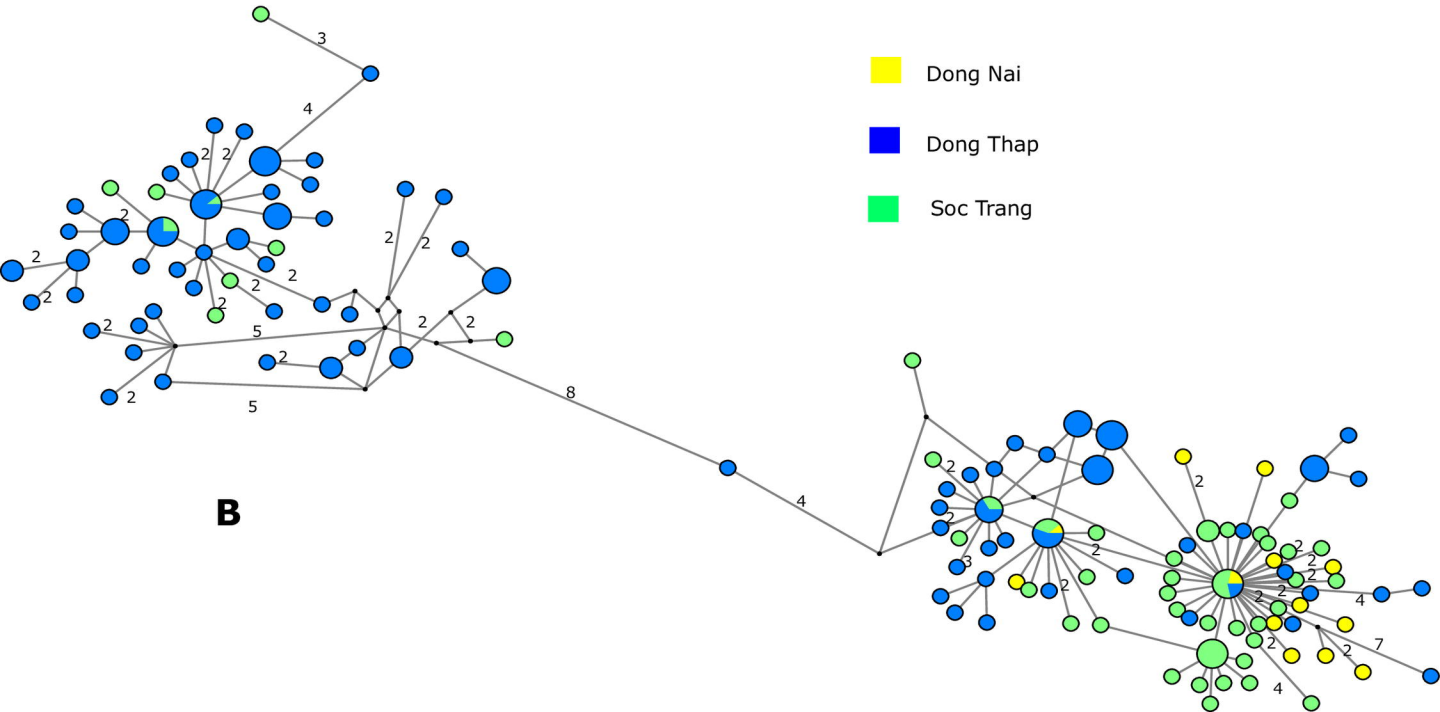
S1 R Code. Code used to conduct the analysis described.


Province status
Sampling
No sampling
Dong Nai
Dong Thap
Soc Trang
Dong Nai
Dong Thap
Soc Trang
Individual count
0
100
200
300
400
trader
large market
restaurant
natural roost
bat guano farm
wildlife farm
Interface
Taxa group
Field Rats
Hystrix brachyura
Rhizomys
Rattus argentiventer
Sciuridae
Microchiroptera
Pteropodidae


Proportion of coronavirus-positive field rats
Proportion
60
40
20
0
20.7%
n=188
32%
n=363
55.6%
n=151
trader
large market
restaurant
Field rat supply chain interface


Scotophilus bat coronavirus 512 2005/PREDICT-VN13F0276
Scotophilus bat coronavirus 512 2005/PREDICT-VN13F0161
Scotophilus bat coronavirus 512 2005/PREDICT-VN14F0014
Scotophilus bat coronavirus 512 2005/PREDICT-VN14F0283
Scotophilus bat coronavirus 512 2005/PREDICT-VN13F0333
Coronaviridae sp. isolate 75-98-L07-R2-CoV-BAT-VN
Scotophilus bat coronavirus 512 2005/PREDICT-VN13F0113
Miniopterus fuliginosus coronavirus isolate CYCU-M22/TW/2013
Rhinolophus monoceros coronavirus isolate CYCU-R14/TW/2013
Bat coronavirus (BtCoV/A535/2005)
Scotophilus bat coronavirus 512 2005/PREDICT-VN13F0065
Coronaviridae sp. isolate 75-98-L07-R4-CoVBAT-VN
CoV-512 Clade
Coronavirus PREDICT CoV-35/GVF-CM-ECO70504
Coronavirus PREDICT CoV-35/CD115889
Bat coronavirus isolate BtCoV/B55080/S.hea/CB/Tha/1/2012
Coronavirus PREDICT CoV-35/VN13F0060
Coronaviridae sp. isolate 75-55-L01-R4-CoV-BAT-VN
Coronavirus PREDICT CoV-35/VN13F0226
Coronavirus PREDICT CoV-35/VN13F0234
Coronavirus PREDICT CoV-35/KHP12PTR1-0160B
Coronaviridae sp. isolate 75-990L01-R5-CoV-BAT-VN
PREDICT_CoV-35 Clade
Bat coronavirus HKU6 PREDICT-EHA-156-12-LS13721
Porcine epidemic diarrhea virus isolate KNU-1708
Bat coronavirus HKU8 isolate HKU8-1
Rousettus bat coronavirus HKU10 isolate 183A
Human coronavirus 229E
NL63-related bat coronavirus strain BtKYNL63-9a
Bat coronavirus HKU2 isolate HKU2-1
Alpha CoV
Bat coronavirus HKU4 isolate HKU4-4
SARS-related coronavirus isolate F23
Coronavirus PREDICT CoV-17/VN13P0020
Bat coronavirus isolate BtCoV/B551005/Pte lyl/CB3-THA/Sep12
Coronavirus PREDICT CoV-17/PB015
Coronavirus PREDICT CoV-17/VN13P0024
Coronavirus PREDICT CoV-17/VN13F0063
PREDICT_CoV-17 Clade
Rousettus bat coronavirus HKU9 PREDICT-KHP13-BN1-0003
Longquan aa coronavirus PREDICT-VN13M0299
Longquan aa coronavirus PREDICT-VN13M0005
Coronaviridae sp. isolate 7562L01R6RATCoV
Coronaviridae sp. isolate 7565L07R5RATCoV
Longquan aa CoV Clade
Betacoronavirus HKU24 strain HKU24-R05010I
Bovine coronavirus isolate BCoV/SLO/5580/2013
Human coronavirus OC43 isolate TNP 12643
Human coronavirus HKU1 isolate SI17244
Murine Coronavirus PREDICT-VN13M0002
Murine Coronavirus PREDICT-VN13M0058
Murine coronavirus KHP13-BLM2-0001
Murine CoV Clade
Beta CoV
Night-heron
Delta CoV
0.20


Hystrix
Microchiroptera
Rattus and Bandicota
Rhizomys
A


Dong Nai
Dong Thap
Soc Trang
B