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Highly diversified coronaviruses in neotropical bats

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Summary

Bats host a broad diversity of coronaviruses (CoVs), including close relatives of human pathogens. There is only limited data on neotropical bat CoVs. We analyzed fecal, blood and intestine specimens from 1,562 bats sampled in Costa Rica, Panama, Ecuador and Brazil for CoVs by broad-range PCR. CoV RNA was detected in 50 bats representing nine different species, both frugivorous and insectivorous. These bat CoVs were unrelated to known human or animal pathogens, indicating an absence of recent zoonotic spill-over events. Based on *RNA-dependent RNA Polymerase* (RdRp)-based grouping units (RGUs) as a surrogate for CoV species identification, the 50 viruses represented five different alphacoronavirus RGUs and two betacoronavirus RGUs. Closely related alphacoronaviruses were detected in *Carollia perspicillata* and *C. brevicauda* across a geographic distance exceeding 5,600 km. Our study expands the knowledge on CoV diversity in neotropical bats and emphasises the association of distinct CoVs and bat host genera.

Introduction

Coronaviruses (CoVs) belong to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* and are enveloped viruses with a positive-sense single-stranded RNA genome. They are classified into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Adams & Carstens, 2012).

In the aftermath of the *severe acute respiratory syndrome* (SARS)-epidemic in 2002/2003 caused by CoV of likely bat origin (Lau *et al.*, 2005), a large number of novel bat CoVs was described (Calisher *et al.*, 2006). The majority of these CoVs originated from African, Asian, and European bats (Chu *et al.*, 2006; Drexler *et al.*, 2011; Drexler *et al.*, 2010; Gloza-Rausch *et al.*, 2008; Pfefferle *et al.*, 2009; Poon *et al.*, 2005; Quan *et al.*, 2010; Tang *et al.*, 2006; Tong *et al.*, 2009). In addition to SARS-CoV, four human coronaviruses (HCoVs), termed HCoV-OC43, -229E, -NL63, and -HKU1 are known (Drosten *et al.*, 2003; Fouchier *et al.*, 2004; Gaunt *et al.*, 2010; Hamre & Procknow, 1966; McIntosh *et al.*, 1967; van der Hoek *et al.*, 2004; Weiss & Navas-Martin, 2005; Woo *et al.*, 2005). Recently, a sixth HCoV was described, causing illness in at least 49 confirmed cases by May, 29th, 2013 (WHO, 29 May 2013; Zaki *et al.*, 2012). Close relatives of this betacoronavirus termed MERS-CoV and of HCoV-229E exist in Old World bats and HCoV-NL63 could be grown in immortalized bat cells (Annan *et al.*, 2013; de Groot *et al.*, 2013; Drexler *et al.*, 2010; Huynh *et al.*, 2012; Lau *et al.*, 2005; Pfefferle *et al.*, 2009), demonstrating the zoonotic potential of previously reservoir-bound bat CoVs. The recent description of a bat CoV related to MERS-CoV in Mexican bats (Anthony *et al.*, 2013) emphasized the relevance of investigating neotropical bats for CoVs.

Bats constitute up to 60% of the local mammalian fauna in pristine neotropical ecosystems and neotropical bats represent nearly 30% of the worldwide bat species (351 out of 1152) (IUCN, 2012; Rex *et al.*, 2008; Schipper *et al.*, 2008). Six of the eighteen extant bat families are endemic to the Neotropics and only three occur both in the New and Old World. The neotropical bats occupy a broad range of ecological niches including insectivorous, nectarivorous, carnivorous, sanguivorous and frugivorous feeding habits (Masters, 2006; Rex *et al.*, 2008; Simmons, 2005a; Teeling *et al.*, 2005).

This species richness contrasts the scarce information on neotropical bat CoVs. There are only two studies on bat CoVs from the Neotropics, one from Trinidad and Tobago yielding two highly diversified alphacoronaviruses (Carrington *et al.*, 2008) and a recently published second one from Mexico yielding alpha and betacoronaviruses summarised in 13 different clades (Anthony *et al.*, 2013). From the neighbouring temperate Northern American areas, additional bat alphacoronavirus clades were described (Dominguez *et al.*, 2007; Donaldson *et al.*, 2010; Huynh *et al.*, 2012; Misra *et al.*, 2009; Osborne *et al.*, 2011).

To increase our knowledge on neotropical CoVs, we analysed 1,866 fecal, blood and intestine specimens from 1,560 individual bats sampled in four neotropical countries. Seven novel alpha- and betacoronavirus clades were detected.

Result and Discussion

The samples comprised 1,868 specimens from 1,562 individual bats collected between 2008 and 2012 in Costa Rica, Panama, Ecuador and Brazil. **Table 1** provides details on the number of specimens per bat species and **Figure 1(a)** shows sampled countries. **Supplementary Table 1** provides the GPS coordinates of specific sampling sites and individual permit numbers. As shown in **Figure 1(b)**, these specimens represented 54 different bat species from seven of the nine families within the phylogeny of neotropical bats. **Table 1** shows that CoV RNA was detected by nested RT-PCR targeting the *RNA-dependent RNA polymerase* (*RdRp*) in 50 specimens from nine different bat species (2.7% of the total samples). In three out of four countries, bat CoVs were detected, while all samples from Ecuador were negative. This was likely due to the smaller sample from this country. All but one of the detections were made in fecal or intestinal tissue specimens (2.8 % of the total fecal/intestinal specimens). Additionally, one blood specimen from an *Artibeus jamaicensis* bat tested positive (0.9% of all blood specimens). No fecal specimen was available from this individual. For all PCR screening amplicons, extension of the partial *RdRp* fragment to 816 nucleotides (nt) was attempted as described previously (Drexler *et al.*, 2010). All CoV sequences were submitted to GenBank under accession numbers

JQ731775-JQ731800 and KC633193-KC633197. **Supplementary Table S2** provides details on accession numbers of individual CoVs.

Results of a Bayesian phylogenetic analysis based on the 816 nt RdRp fragment are given in **Figure 2(a).** Because this larger fragment was not available for most previously described CoVs and could not be obtained for two alphacoronaviruses from this study, the 404 nt *RdRp* fragment generated by our and most other published CoV screening assays was also analysed. **Figure 2(b)** shows the phylogeny of this shorter fragment for the genus *Alphacoronavirus*. The novel bat CoVs clustered as eight independent branches in the *Alpha*- and *Betacoronavirus* genera and were unrelated to any known Old World bat CoV. **Table 2** shows the high diversification within the neotropical bat CoVs which ranged from 6.6-37.5% amino acid sequence distance in the 816 nt *RdRp* fragments. As shown in **Table 3**, the novel CoVs were also unrelated to any known CoV from humans or other animals, with amino acid sequence distances ranging from 12.1-39.0% in comparison to all defined CoV species. This contrasted with Old World bat CoVs for which zoonotic transmission to humans likely occurred, exemplified by SARS-related viruses in rhinolophid bats in Asia (Drexler *et al.*, 2010; Lau *et al.*, 2005) or HCoV-229E-related viruses in hipposiderid bats in Africa (Pfefferle *et al.*, 2009).

We previously proposed a simplified CoV classification into RdRp-based grouping units [RGU] separated by >4.8% amino acid (aa) distance for alphacoronaviruses and >6.3% for betacoronaviruses in the translated 816 nt *RdRp* fragment (Drexler *et al.*, 2010). Based on these criteria, the novel CoVs could be classified as five *Alphacoronavirus* RGUs and two *Betacoronavirus* RGUs. Four of these five *Alphacoronavirus* RGUs were previously undefined and originated from bat species belonging to the genera *Phyllostomus*, *Artibeus* and *Anoura* in the Phyllostomidae family (shown according to the countries of origin in green and orange in **Figure 2(a)**). In addition, the RGU defined by a previously described CoV from *Carollia perspicillata* from Trinidad and Tobago (Carrington *et al.*, 2008) was extended by novel *C. brevicauda* and *C. perspicillata* CoVs from Costa Rica and Brazil (shown in orange and pale blue in **Figure 2(a)**). As shown in **Table 2**, the amino acid sequence distance within all known alphacoronaviruses from *Carollia* bats was only 1.1%. Another novel *Alphacoronavirus*

clade could be detected in *Molossus rufus* and *M. currentium* from Brazil (shown in pale blue in **Figure 2(b))**. An RGU could not be defined because only the 404 nt *RdRp* fragment was available. Still, these two viruses differed by 6.8% aa distance within this smaller sequence fragment, indicating they might constitute two separate RGUs. The novel *Betacoronavirus* RGUs differed from each other by 14.0-14.3% aa sequence distance and were defined by CoVs detected in samples from *Pteronotus parnellii* and *C. perspicillata* (shown in orange in **Figure 2(a)**).

A recent study on bat CoVs from Mexico (Anthony *et al.*, 2013) yielded alpha and betacoronaviruses summarised in 13 different clades whose phylogenetic position indicated relatedness to some of the bat CoVs described in this study. Because only short sequence fragments of 243 to 297 nt were available for these bat CoVs and because these sequences did not overlap with our *RdRp* fragments, these CoVs could not be included into our phylogenetic analyses. Still, consideration of the phylogeny and the bat hosts of these Mexican CoVs could indicate that some of these viruses were related to three of the nine RGUs we describe in this study, including the *Carollia* and *Artibeus* alphacoronavirus RGUs and the *Pteronotus* betacoronavirus RGU.

Some bat species are widely distributed across the Neotropics. As illustrated in **Figure 3**, closely related alphacoronaviruses were detected in *C. perspicillata* from Brazil and Costa Rica, 5,600 km apart. Since *C. perspicillata* does not migrate over long distances (Fleming & Heithaus, 1986; Kunz & Fenton, 2003), recent transmission events are unlikely to explain these findings. Interestingly, the same virus was also detected in other *Carollia* species including *C. brevicauda* and possibly related CoV sequences from *C. sowelli* and *C. perspicillata* from Mexico (Anthony *et al.*, 2013). This was compatible with SARS-related CoVs in different rhinolophid bat species from Europe and China and with alphacoronaviruses detected in vespertilionid bats of one genus across geographic distances exceeding 2,000 km (Drexler *et al.*, 2010; Tang *et al.*, 2006). The host genus, rather than the host species, may therefore define the habitat of CoV species (Drexler *et al.*, 2010).

In addition to local species richness, ecological host factors such as feeding and roosting habits (Drexler *et al.*, 2011) and contacts to other animals in the ecosystem likely influence the diversification and occurrence of bat CoVs (Parrish *et al.*, 2008). For example, closely related viruses were detected in nectarivorous *Glossophaga soricina* described previously (Carrington *et al.*, 2008) and omnivorous *Phyllostomus discolor* from our study (3.8% aa distance in the 404 nt *RdRp* fragment). *P. discolor* is mainly nectarivorous and visits some of the same flowers as *G. soricina* (Kwiecinski, 2006), which may facilitate hypothetical exchange of viruses between the two bat genera. Sporadic observations of closely related CoVs in different bat species and even families were shown previously by us (Drexler *et al.*, 2010) and other groups (Anthony *et al.*, 2013; Lau *et al.*, 2010). It remains unclear whether ecological factors like population density and feeding habits influence the exchange of viruses between different bat species and the co-segregation of hosts and viruses.

No bat CoVs closely related to HCoV-OC43, -HKU1 and -NL63 have so far been found. Huynh et al. recently described alphacoronaviruses from Northern American bats that showed 12.9-17.6% aa distance to HCoV-NL63 in a translated 675 nt fragment partially overlapping with the *RdRp* sequences generated in our study (Huynh *et al.*, 2012). Previous detections of alphacoronaviruses in Old World bats showed even lower sequence distances to HCoV-NL63 in the same 675 nt *RdRp* fragment, exemplified by 11.5% aa distances of *Miniopterus* and *Nyctalus* bat CoVs (Drexler *et al.*, 2010). Furthermore, bat CoVs closely related to the other human alphacoronavirus, HCoV-229E, exist in African *Hipposideros* bats (Pfefferle *et al.*, 2009). These data jointly highlight the possibility that bat CoVs closely related to HCoV-NL63 may exist, but are yet to be described.

The nearly complete absence of neotropical bat CoVs more closely related to human pathogens could be due to lower chances of transmission, such as rare consumption of bats as bush meat in the New World in contrast to the Old World tropics (Mickleburgh *et al.*, 2009; Setz & Sazima, 1987). However, the growing invasion and destruction of neotropical habitats (Dale *et al.*, 1994; Kolb & Galicia, 2011) may provide further exposure of humans to bats and their viruses, as exemplified by the emergence of Nipah virus in 1998 (Daszak *et al.*, 2001; Keesing *et al.*, 2010). The recent

identifications of betacoronaviruses related to MERS-CoV in Mexican *Nyctinomops* (Anthony *et al.*, 2013), European *Pipistrellus* and African *Nycteris* (Annan *et al.*, 2013) bats highlight the relevance of studying the diversity of CoVs in bat reservoirs. It is important to note that actions aiming at eradication of bats as potential virus hosts may disrupt important ecological functions, e.g., pollination and natural pest reduction (Cleveland *et al.*, 2006; Kalka *et al.*, 2008).

The diversified ecology, a high number of co-existing bat species and their local abundance in relation to other mammalian species (Rex *et al.*, 2008), could make neotropical bats a leading receiver and spreader of viruses in neotropical ecosystems. For example, sanguivorous bats only exist in the Neotropics and could hypothetically facilitate viral host switches between bats and other mammals. This is exemplified by the detection of a small sequence fragment of bovine CoV in vampire bat feces (Brandao *et al.*, 2008), which could hypothetically result from feeding on cattle. Further studies on bat CoV could therefore focus on animals with close bat contact, such as prey of vampire bats or feline, canine and non-human primate bat predators (Delpietro *et al.*, 1994; Rodriguez-Duran *et al.*, 2010; Souza, 1997; Taylor & Lehman, 1997).

Methods

We declare that all sampling and capture of wild animals as well as sample transfers were done with the proper wildlife permits and ethics clearances and complied with the current laws of host countries. Sampling was performed between 2008 and 2012 at 44 different sites in four countries (**Figure 1**). The complete geographic coordinates of all sampling sites and corresponding sampling permits are given in supplementary **Table S1**. In Brazil and Costa Rica, bats were mainly caught in front of caves, while bat catching in Ecuador and Panama focused mainly in neotropical forests. No bat species were specifically targeted. In Costa Rica and Ecuador, bats were caught using mist nets and kept in individual cotton bags for a few minutes until examination. Fecal pellets produced in the meantime were taken directly from individual bags and stored in 500 µl of RNAlater RNA stabilization solution (Qiagen, Hilden, Germany) until further processing. 50 µl of the supernatants were suspended into 560

µl of Buffer AVL from the Viral RNA mini kit (Qiagen) and processed according to the manufacturer's instructions. Blood samples were taken from Panamanian bats for an ecological study on blood parasites (Cottontail *et al.*, 2009). Depending on the available quantity, up to 50µL of blood was extracted likewise. For some of the Panamanian bats sampled in 2011, fecal samples were additionally available and processed as described above. The Brazilian specimens were sampled during activities on prevention of rabies (Carneiro *et al.*, 2010). Bats were caught at roosts using mist nets, killed with ether and transported on ice to the laboratory where bats were typed and dissected. Approximately 30 mg of intestinal tissue was homogenized in a bead mill, followed by extraction of RNA using the RNeasy Kit (Qiagen). Elution volumes were 50µL for fecal and blood specimens and 100µL for tissue specimens.

Reverse-transcription polymerase chain reaction (RT-PCR) covering the subfamily *Coronavirinae* was done as described previously (de Souza Luna *et al.*, 2007). The 455 base pair amplicons from the *RNA-dependent RNA polymerase* (*RdRp*) generated by the screening RT-PCR (404 nucleotides (nt) after exclusion of PCR primers) were extended towards the 5'-end of the genome using virus-specific reverse primers and upstream consensus forward primers, as described previously (Drexler *et al.*, 2010). Translated nucleic acid alignments containing the novel viruses and CoV reference strains were done using the BLOSUM algorithm in the Mega5 software package (Tamura *et al.*, 2011). The final datasets used for phylogenetic analyses consisted of 816 and 404 nt gap-free coding *RdRp* alignments. Bayesian phylogenies were conducted with MrBayes V3.1 using the translated nucleotide sequences and a WAG amino acid substitution model over 4,000,000 generations sampled every 100 steps. The resulting 40,000 final trees were annotated using a burn-in of 10,000 in TreeAnnotator V1.5 and visualized with FigTree V1.4 from the BEAST package (Drummond & Rambaut, 2007; Ronquist & Huelsenbeck, 2003).

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Table 1. Bat species tested for coronaviruses

Family	Species	No. of samples			Total PCR positive (%) per specimen	Sampling site (year)[‡] *positive location/year
		Feces	Blood	Intestine		
Emballonuridae	*Peropteryx kappleri*	5				CRC(2010)
	Rhynchonycteris naso	1				ECU(2010)
	Saccopteryx bilineata	114				PAN(2010/2011); CRC(2010)
	Saccopteryx leptura	1				PAN(2011)
Phyllostomidae	*Anoura geoffroyi*	101			4 (3.96) feces	CRC(2010*)
	Artibeus jamaicensis[†]	295	68		3 (1.02) feces	PAN(2010*/2011); ECU(2010)
	Artibeus lituratus	41	13		1 (2.44) feces 1 (7.69) blood	PAN(2010*/2011*); ECU(2010)
	Artibeus obscurus	2				ECU(2010)
	Artibeus phaeotis	4	1			PAN(2011)
	Artibeus watsoni	2	2			PAN(2011)
	Carollia brevicauda	3		104	6 (5.77) intestine	BRA(2009*);ECU(2010)
	Carollia castanea	34	11			PAN(2010/2011); CRC(2010); ECU(2010)
	Carollia perspicillata	283	1	175	14 (8.00) intestine 7 (2.47) feces	BRA(2009*); CRC(2010*/2011*/2012*); PAN(2010/2011); ECU(2010)
	Carollia spec.			15		BRA(2009)
	Chrotopterus auritus	1				ECU(2010)
	Desmodus rotundus		1	29		BRA(2008/2009); PAN(2011)
	Enchisthenes hartii	3				CRC(2010)
	Glossophaga commissarisi	3				CRC(2010)
	Glossophaga soricina	47		2		BRA(2009); PAN(2010/2011); CRC(2010/2012)
	Lampronycteris brachyotis	2				CRC(2012)
	Lonchorhina aurita			1		BRA(2009)
	Lonchophylla robusta	1				CRC(2012)
	Lophostoma brasiliense	2				PAN(2011)
	Lophostoma silvicolum	25	2			PAN(2010/2011); ECU(2010)
	Mesophylla macconnellii	1				ECU(2010)
	Micronycteris hirsuta	3				PAN(2010/2011)
	Micronycteris microtis	6				PAN(2010/2011)
	Micronycteris minuta	1	1			PAN(2011)
	Mimon crenulatum	10	1			PAN(2010/2011); ECU(2010)
	Phylloderma stenops	1	2			PAN(2011)
	Phyllostomus discolor	10			2 (20.00) feces	PAN(2011*)
	Phyllostomus hastatus	5				PAN(2010/2011); ECU(2010)
	Phyllostomus elongatus	4				ECU(2010)
	Platyrrhinus brachycephalus	1				ECU(2010)
	Platyrrhinus helleri	1				PAN(2010)
	Platyrrhinus infuscus	2				ECU(2010)
	Rhinophylla pumilio	4				ECU(2010)
	Sturnira lilium	3				ECU(2010)
	Sturnira magna	3				ECU(2010)
	Tonatia saurophila	7				PAN(2010/2011); ECU(2010)

	Trachops cirrhosus	12	1	1		BRA(2009); PAN(2010/2011); ECU(2010)
	Uroderma bilobatum	32				PAN(2010/2011)/ ECU(2010)
	Vampyressa bidens	1				ECU(2010)
	Vampyressa thyone		1			PAN(2011)
	Vampyrodes caraccioli	5	4			PAN(2011)
Mormoopidae	*Pteronotus parnellii*	290	6	4	10 (3.45) feces	PAN(2010/2011); CRC(2010*/2011*/2012*)
Noctilionidae	*Noctilio leporinus*	1				PAN(2011)
Vespertilionidae	*Myotis albescens*	2				ECU(2010)
	Myotis nigricans	7				PAN(2010/2011); ECU(2010)
	Rhogeessa tumida	3				PAN(2010); CRC(2012)
Molossoidea	*Eumops maurus*	1				ECU(2010)
	Molossus currentium			10	1 (10.00) intestine	BRA(2009*)
	Molossus molossus	1		1		BRA(2009); PAN(2010)
	Molossus rufus	2		17	1 (5.88) intestine	BRA(2009*); ECU(2010)
Natalidae	*Natalus lanatus*	5				CRC(2010/2012)
Total	**54 species 1,562 individual bats**	**1,394**	**115**	**359**	**50 (2.68)**	

‡ CRC= Costa Rica, ECU=Ecuador, PAN= Panama, BRA= Brazil

† including 3 negative samples from *A. planirostris* summarized under *A. jamaicensis* according to (Simmons, 2005b).

Table 2. Amino acid identities within neotropical bat coronaviruses

	Percentage amino acid identity across 272 amino acids within the translated 816 nucleotide fragment†						
CoV RGU/clade	**Carollia α-CoV‡**	**Phyllostomus α-CoV**	**Artibeus α-CoV I**	**Anoura α-CoV**	**Artibeus α-CoV II**	**Pteronotus β-CoV**	**Carollia β-CoV**
Carollia α-CoV‡	98.9-100	92.3-93.4	86.4-86.8	85.7-86.4	83.5-83.8	66.9-67.6	65.1
Phyllostomus α-CoV		98.9-100	88.2-89.0	84.9-85.7	82.7-83.5	65.1-65.8	62.5-62.9
Artibeus α-CoV I			99.6-100	83.8	83.8-84.2	64.7-65.1	63.2
Anoura α-CoV				100	90.1	66.9	63.2
Artibeus α-CoV II					100	67.3-67.6	66.2
Pteronotus β-CoV						99.3-100	85.7-86
Carollia β-CoV							100

480 †Analyses were conducted in MEGA5 (Tamura *et al.*, 2011) using the pairwise deletion option.
‡ including EU769557 described by (Carrington *et al.*, 2008)

Table 3. Amino acid identities of neotropical bat coronaviruses with designated CoV species

	Percentage amino acid identity across 272 amino acids within the translated 816 nucleotide fragment[a]							
	Alpha CoV1[c]	**PEDV[d]**	**Scotophilus 512 BtCoV[e]**	**HKU2[f]**	**229E[g]**	**NL63[h]**	**HKU8[i]**	**Miniopterus BtCoV 1[j]**
Carollia α-CoV[b]	77.6-78.3	85.3-85.7	82.7-83.5	79-79.4	80.1-80.9	83.5-84.2	84.6-85.3	83.8-84.2
Phyllostomus α-CoV	76.8-77.9	83.5-84.2	81.6-82	78.7-78.7	81.6-82.4	82.0-82.7	85.3-86	84.9-85.7
Artibeus α-CoV I	78.3-78.7	84.2-84.6	82.7-83.1	79.8	80.5-80.9	82.7-83.1	84.2	84.2
Anoura α-CoV	87.9-79.4	83.8	82	80.9	80.9	83.8	87.1	87.9
Artibeus α-CoV II	80.1-80.5	86	83.8	79.8	79.8	82	84.2	84.6
Pteronotus β-CoV	70.2-71	68.4-68.7	66.9-67.3	68.4-68.7	64.7-65.1	65.1-65.4	66.5-66.9	64-64.3
Carollia β-CoV	68.4-68.7	66.5	65.8	66.5	62.9	61	63.2	64

	BetaCoV 1[k]	**HKU1[l]**	**Murine CoV[m]**	**HKU4[n]**	**HKU5[o]**	**HKU9[p]**	**SARS related[q]**	**MERS-CoV[r]**
Carollia α-CoV	62.1-62.5	61.8	61.8-62.1	64.3-64.7	64.0-64.7	63.6-64.7	63.6-64.3	65.1-65.4
Phyllostomus α-CoV	62.1-62.9	61.8-61.4	62.1-62.5	62.5-62.9	62.5-62.9	63.6-65.1	62.9-63.6	63.2-63.6
Artibeus α-CoV I	62.5	61	61	61.8	61.8	62.5-63.6	61.8-62.1	61.8
Anoura α-CoV	64-64.3	63.6	62.9	63.6	64.7	62.5-63.2	63.6	63.6
Artibeus α-CoV II	64.7-65.1	64	63.2	64.3	65.1	63.2-64.0	66.2-66.5	64.3
Pteronotus β-CoV	75-75.7	74.3-74.6	73.2-73.5	77.2-77.6	77.2-77.6	79.4-80.1	77.6-77.9	76.8-77.2

Carollia β-CoV	73.5-73.9	73.5	73.2	76.5	77.2	76.8-77.2	77.2-77.9	77.2

[a]Analyses were conducted in MEGA5 (Tamura *et al.*, 2011) using the pairwise deletion option.
[b]including EU769557 described by (Carrington *et al.*, 2008)
GenBank accession numbers:
[c]DQ010921, DQ811789 Alpha CoV1
[d]AF353511 PEDV
[e]DQ648858 Scotophilus 512 BtCoV
[f]EF203064 HKU2
[g]AF304460 229E
[h]AY567487, AY518894 NL63
[i]GU190248 HKU8
[j]EU420138 Miniopterus BtCoV 1
kAY585228, U00735 BetaCoV 1
[l]DQ415914 HKU1
[m]FJ647225 Murine CoV
[n]EF065505 HKU4
[o]EF065509 HKU5
[p]EF065513, EF065515 HKU9
[q]DQ022305, AY274119 SARS related
[r]JX869059 MERS-CoV

Figure legends

Figure 1. Sampling sites and bat phylogeny

(a)The bat samples used in this study and their countries of origin are listed according to their species and families, with Coronavirus-positive bat species additionally named. (b) Chiropteran phylogeny adapted from (Simmons, 2005a). Bat families only distributed in the Old World are shown in grey, those endemic to the Neotropics are printed boldface and those distributed in both the New and the Old World are marked with an asterisk. Families included in the analyses are framed with grey boxes and families testing positive for CoV in this study are given in red.

Figure 2. RdRp-based phylogeny including novel bat coronaviruses

Bayesian phylogenies of translated 816-nt (a) and 404-nt (b) gap-free RNA-dependent RNA-polymerase (RdRp) gene sequence fragments. For (a), a whale gammacoronavirus and for (b), Transmissible gastroenteritis virus of swine (TGEV) were used as outgroups. For clarity of presentation, only posterior probability values above 0.7 are shown and values at crown positions were removed. Novel bat coronaviruses from this study are colored according to their country of origin (pale blue=Brazil; orange=Costa Rica; green=Panama). New World bat coronaviruses described previously are shown in boldface. Taxa are named according to the following pattern: identification code/strain or isolate/typical host/country/collection year/accession number. The right-hand columns show novel Bat-CoV RGUs and designated CoV species used for further analysis. Car bre, *Carollia brevicauda*; Car per, *Carollia perspicillata*; Phy dis, *Phyllostomus discolor*; Art jam, *Artibeus jamaicensis*; Art lit, *Artibeus lituratus*; Min sch, *Miniopterus schreibersii*; Min pus, *Miniopterus pusillus*; Min tri, *Miniopterus tristis*; Min mag, *Miniopterus magnater*; Min inf, *Miniopterus inflatus*; Nyc lei, *Nyctalus leisleri*; Ano geo, *Anoura geoffroyi*; Rhi bla, *Rhinolophus blasii*; Rhi fer, *Rhinolophus ferrumequinum*; Myo dau, *Myotis daubentonii*; Myo ric, Myotis ricketti; Sco kuh, *Scotophilus kuhlii*; Sus scr, *Sus scrofa*; Cha sp., *Chaerephon* sp.; Hip sp, *Hipposideros* sp.; Hom sap, *Homo sapiens*; Rhi sin, *Rhinolophus sinicus*; Rhi eur, *Rhinolophus euryale*; Mus vis, *Mustela vison*; Hip com, *Hipposideros commersoni*; Fel sil, *Felis silvestris*; Rou les, *Rousettus leschenaulti*; Rou aeg, *Rousettus aegyptiacus*; Pte par, *Pteronotus parnellii*; Pip abr, *Pipistrellus abramus*; Tyl pac, *Tylonycteris pachypus*; Bos pri, *Bos primigenius*; Mus mus, *Mus musculus*; Del leu, *Delphinapterus leucas*; Glo sor, *Glossophaga soricina*; Myo occ, *Myotis occultus*; Rhi meg, *Rhinolophus megaphyllus*; Min aus, *Miniopterus australis*; Mol ruf, *Molossus rufus*;

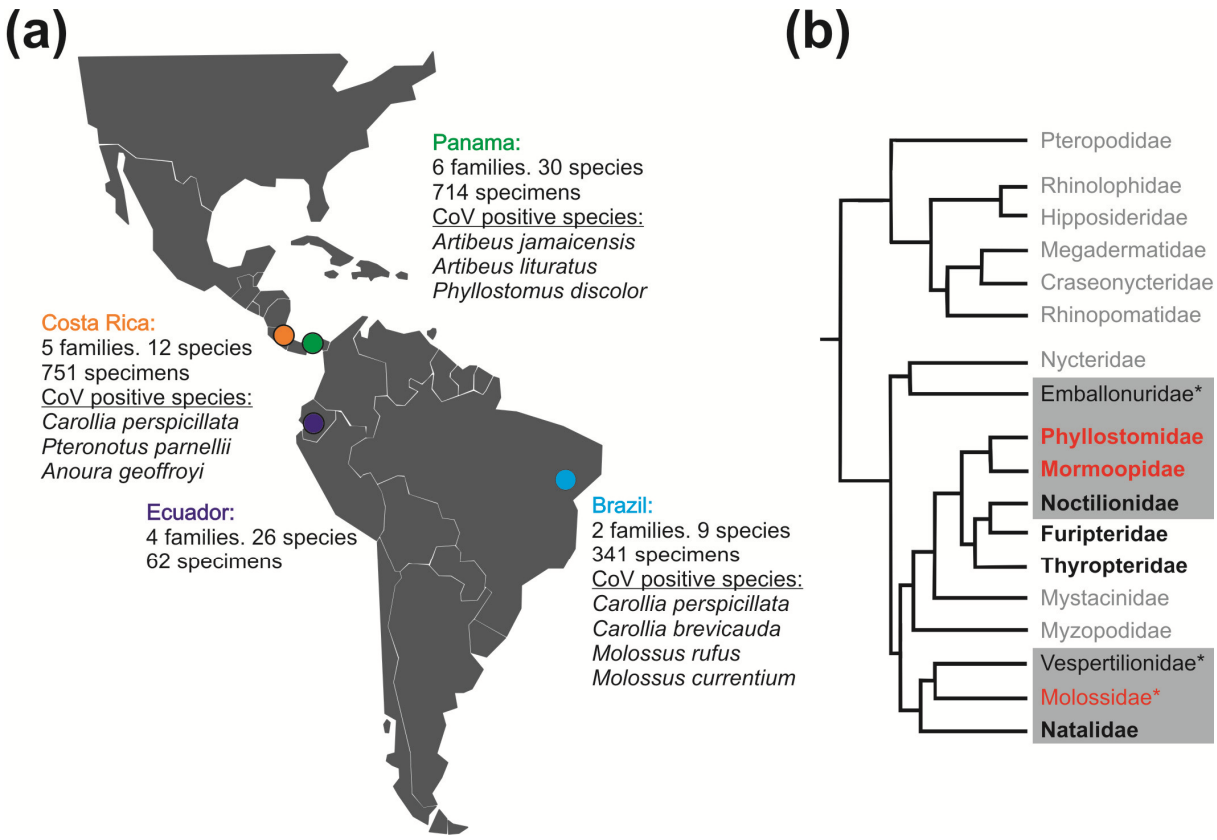
Figure 3. *Carollia perspicillata* distribution and detection of related alphacoronaviruses

(a) Distribution of *Carollia perspicillata* adapted from the IUCN red list (IUCN, 2012) is shown in grey. Sampling sites with detection of *Carollia* alphacoronaviruses are marked with dots (b) Extract of

the coronavirus phylogeny shown in Figure 2 representing the *Carollia* alphacoronavirus clade, including two viruses from Brazilian *C. brevicauda*. CoV amino acid identities between Trinidad and Tobago, Panama and Brazil are shown next to the brackets.

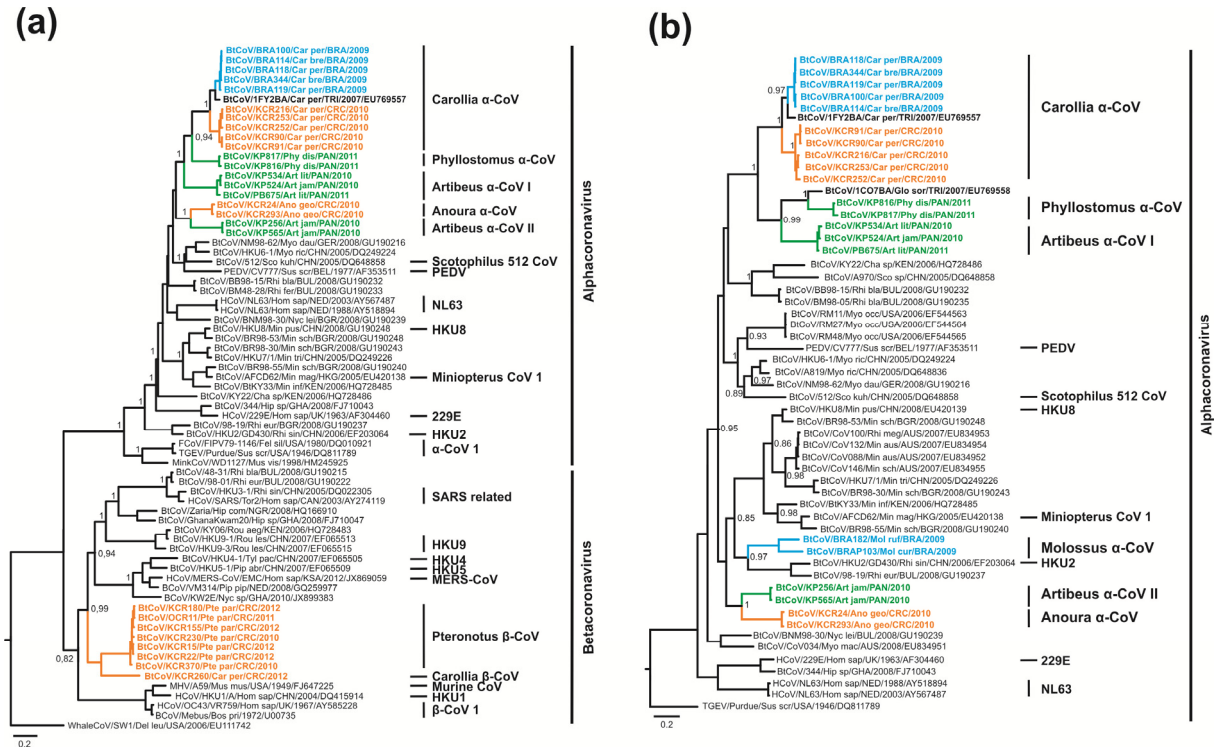
(c) *C. perspicillata* caught in Costa Rica (Photo by A. R.).

Figure 1. Sampling sites and bat phylogeny



555

Figure 2. RdRp-based phylogeny including novel bat coronaviruses



560 **Figure 3. *Carollia perspicillata* distribution and detection of related alphacoronaviruses**