Coronaviruses in bats from Mexico

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Bats are reservoirs for a wide range of human pathogens including Nipah, Hendra, rabies, Ebola,
Marburg and severe acute respiratory syndrome coronavirus (CoV). The recent implication of a
novel beta (β)-CoV as the cause of fatal respiratory disease in the Middle East emphasizes the
importance of surveillance for CoVs that have potential to move from bats into the human
population. In a screen of 606 bats from 42 different species in Campeche, Chiapas and Mexico
City we identified 13 distinct CoVs. Nine were alpha (α)-CoVs; four were β-CoVs. Twelve were
novel. Analyses of these viruses in the context of their hosts and ecological habitat indicated that
host species is a strong selective driver in CoV evolution, even in allopatric populations separated
by significant geographical distance; and that a single species/genus of bat can contain multiple
CoVs. A β-CoV with 96.5 % amino acid identity to the β-CoV associated with human disease in
the Middle East was found in a Nyctinomops laticaudatus bat, suggesting that efforts to identify
the viral reservoir should include surveillance of the bat families Molossidae/Vespertilionidae, or
the closely related Nycteridae/Emballonuridae. While it is important to investigate unknown viral
diversity in bats, it is also important to remember that the majority of viruses they carry will not
pose any clinical risk, and bats should not be stigmatized ubiquitously as significant threats to
public health.

INTRODUCTION

Coronaviruses (CoVs), in the subfamily Coronavirinae, are
enveloped, single-stranded positive-sense RNA viruses with
spherical virions of 120–160 nm (King et al., 2012). They are
among the largest RNA viruses, with complex polyadenyl-
ated genomes of 26–32 kb, and are divided into four genera:
Alphacoronavirus (α-CoV) and Betacoronavirus (β-CoV)
(infecting mainly mammals), and Gammacoronavirus (γ-CoV)
and Deltacoronavirus (δ-CoV) (infecting mainly birds)
(King et al., 2012; Woo et al., 2012). Infection with CoVs is
often asymptomatic, however, they can be responsible for
a range of respiratory and enteric diseases of medical and
veterinary importance. Chief among these is the severe acute
respiratory syndrome (SARS)-CoV, which caused a pand-
emic in 2002–2003. This outbreak lasted for 8 months,
infected 8096 people and resulted in 774 deaths (WHO,
2004). Since then, a renewed public health interest in these
viruses has been stimulated by the emergence of a novel β-
CoV in nine people from the Middle East. In these cases the
patients suffered from acute, serious respiratory illness,
presenting with fever, cough, shortness of breath and
difficulty breathing (Zaki et al., 2012). Five cases later died.
It is currently unclear where this particular virus came from, though genomic analyses have shown similarity to bat CoVs (Zaki et al., 2012). Given that the majority of emerging pathogens are known to originate in animals (Jones et al., 2008), the concern is that this current outbreak may represent a further example of zoonotic transmission from wildlife to people, though further ecological, immunological and evolutionary information is still required to confirm this.

Knowledge about CoV diversity has increased significantly since the SARS pandemic, with the description of several novel viruses from a wide range of mammalian and avian hosts (Cavanagh, 2005; Chu et al., 2011; Dong et al., 2007; Felippe et al., 2010; Guan et al., 2003; Jackwood et al., 2012; Lau et al., 2012b; Woo et al., 2009a, b, 2012). Bats in particular seem to be important reservoirs for CoVs, and discovery efforts have been increasingly focused on them since the recognition of SARS-like CoVs in rhinolophid species (Lau et al., 2005; Li et al., 2005) and because bats appear to be reservoirs for a large number of other viruses (Calisher et al., 2006; Drexler et al., 2012; Jia et al., 2003; Leroy et al., 2005; Rahman et al., 2010; Towner et al., 2007). Several of the novel CoVs described in the last decade were identified in bats of various species and demonstrate a strong association between bats and CoVs (August et al., 2012; Carrington et al., 2008; Chu et al., 2009; Dominguez et al., 2007; Drexler et al., 2011, 2010; Falcón et al., 2011; Ge et al., 2012b; Gloza-Rausch et al., 2008; Li et al., 2005; Misra et al., 2009; Osborne et al., 2011; Pfefferle et al., 2009; Quan et al., 2010; Reusken et al., 2010; Tong et al., 2009b; Woo et al., 2006; Yuan et al., 2010).

The large number of CoVs that continue to be described in bats suggest that many (if not most) bat species might be associated with at least one CoV. Given that there are ~1200 extant bat species known, the existence of an equally large diversity of CoVs must be considered likely. Initially, most discovery effort was targeted towards bats from China (Ge et al., 2012a; Lau et al., 2010b; Li et al., 2005; Tang et al., 2006; Woo et al., 2006), followed by limited surveillance in other South-east Asian countries including Japan, the Philippines and Thailand (Gouilh et al., 2011; Shirato et al., 2012; Watanabe et al., 2010). In the Old World, novel CoVs have been found in both Europe and Africa (August et al., 2012; Drexler et al., 2011, 2010; Gloza-Rausch et al., 2008; Pfefferle et al., 2009; Quan et al., 2010; Reusken et al., 2010; Rihtaric et al., 2010; Tong et al., 2009b).

In contrast, very few investigations have been conducted in the New World and little is known about the diversity of CoVs found here. Dominguez et al. (2007) were the first to test bats in the New World for CoV, followed by Donaldson et al. (2010) and Osborne et al. (2011). These groups tested bats captured in Colorado and Maryland and found α-CoVs from five different species of evening bats (Eptesicus fuscus, Myotis evotis, Myotis lucifugus, Myotis occultus and Myotis volans). All were unique compared with CoVs found in Asia. Misra et al. (2009) then tested M. lucifugus samples from Canada and detected a similar α-CoV to those found in myotis bats from Colorado. In South America, Carrington et al. (2008) identified an α-CoV in two species of leaf-nosed bats, Carollia perspicillata and Glossophaga soricina, which clustered most closely with CoVs from North American and European bats.

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**Fig. 1.** (a) Map of sampling sites, (i) D.F., Mexico City, (ii) Reserva de la Biosfera Montes Azules, Chiapas, (iii) the Reserva de la Biosfera Calakmul, Campeche. (b) CoV-positive bat, species Lonchorhina aurita. This individual (PMX-505) was positive for the novel α-CoV Mex_CoV-3.
Table 1. Summary of all bats captured at each site

A total of 606 bats were sampled across three sites. The number of CoV PCR positives (Pos) are indicated in parentheses, together with the number of CoV clades in square brackets. All bat species captured are endemic to the Americas.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Trophic guild</th>
<th>Campeche (# CoV PCR Pos)/[CoV clade(s)]</th>
<th>Chiapas (# CoV PCR Pos)/[# CoV clade(s)]</th>
<th>D.F. Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Undisturbed</td>
<td>Disturbed</td>
<td>Undisturbed</td>
</tr>
<tr>
<td>Phyllostomidae</td>
<td>Artibeus lituratus</td>
<td>Frugivorous</td>
<td>26 (4) [5b, 11b]</td>
<td>28 (1) [11a]</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Artibeus phaeotis</td>
<td>Frugivorous</td>
<td>21 (1) [11b]</td>
<td>3</td>
<td>3 (2) [11a, 11b]</td>
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<tr>
<td></td>
<td>Artibeus jamaicensis</td>
<td>Frugivorous</td>
<td>33 (2) [5b]</td>
<td>23 (1) [4]</td>
<td>17 (2) [5a]</td>
</tr>
<tr>
<td></td>
<td>Artibeus watsoni</td>
<td>Frugivorous</td>
<td>5</td>
<td>3</td>
<td>7</td>
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<tr>
<td>Glossophaga</td>
<td>Glossophaga soricina</td>
<td>Nectivorous</td>
<td>1</td>
<td>8</td>
<td>12</td>
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<tr>
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<td></td>
<td>Carollia sowelli</td>
<td>Frugivorous</td>
<td>27</td>
<td></td>
<td>14 (4) [1, 2, 5b]</td>
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<td>Frugivorous</td>
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<td>4 (2) [1]</td>
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<td>7</td>
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<td>Sturnira lilium</td>
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<td>6</td>
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<td></td>
<td>Leptonycteris yerbabuenae</td>
<td>Nectivorous</td>
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<tr>
<td></td>
<td>Centurio senex</td>
<td>Frugivorous</td>
<td>1</td>
<td>3</td>
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<td></td>
<td>Platyrrhinus helleri</td>
<td>Frugivorous</td>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td></td>
<td>Uroderma bilobatum</td>
<td>Frugivorous</td>
<td></td>
<td></td>
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<td></td>
<td>Desmodus rotundus</td>
<td>Haematophagous</td>
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<tr>
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<td>Micronycteris schmidtorum</td>
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<td>Frugivorous</td>
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<tr>
<td></td>
<td>Choeronyctes godmani</td>
<td>Nectivorous</td>
<td></td>
<td></td>
<td>6</td>
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<tr>
<td>Trachops cirrhosus</td>
<td>Carnivorous</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Tonatia saurophila</td>
<td>Insectivorous</td>
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<td>1</td>
<td></td>
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<tr>
<td>Chrotopterus auritus</td>
<td>Carnivorous</td>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>Lonchorhina aurita</td>
<td>Insectivorous</td>
<td>1 (1) [3]*</td>
<td></td>
<td>1</td>
<td></td>
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<tr>
<td>Phyloderma stenops</td>
<td>Frugivorous</td>
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<tr>
<td>Mormoopidae</td>
<td>Mormoops megaslopha</td>
<td>Insectivorous</td>
<td>9</td>
<td></td>
<td>2</td>
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<tr>
<td></td>
<td>Pteronotus davii</td>
<td>Insectivorous</td>
<td></td>
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<td>1</td>
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<td></td>
<td>Pteronotus parnelli</td>
<td>Insectivorous</td>
<td>1 (1) [10]</td>
<td>1</td>
<td>10</td>
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<td>Nyctinomops macrotis</td>
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<tr>
<td></td>
<td>Nyctinomops laticaudatus</td>
<td>Insectivorous</td>
<td>5 (1) [9]</td>
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<tr>
<td></td>
<td>Tadarida brasiliensis</td>
<td>Insectivorous</td>
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<tr>
<td>Vespertilionidae</td>
<td>Myotis velifer</td>
<td>Insectivorous</td>
<td></td>
<td></td>
<td>7 (3) [7]</td>
</tr>
<tr>
<td></td>
<td>Myotis occultus</td>
<td>Insectivorous</td>
<td></td>
<td></td>
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</tbody>
</table>
Nothing is known about the diversity of CoVs in Mexico. Many of the bats studied in Canada and the USA are also found in Mexico, yet it is unknown whether similar viruses are found here. It is also unknown whether $\beta$-CoVs exist in the Americas, or whether the $x$-CoVs predominate. This is a substantive gap in our knowledge of CoV ecology because one-third of all bat species (and 75% of all known bat genera) are found in the neotropics, which includes southern Mexico (Osborne et al., 2011; Wilson & Reeder, 2005). It seems probable that the high ecological, trophic and taxonomic diversity found in Neotropical and Nearctic bats in Mexico (Arita & Ortega, 1998) would be matched by an equally diverse population of novel CoVs. In this study we examined 42 species of bats using broadly reactive consensus PCR for the discovery of novel CoVs, and found an additional 13 viral lineages/clades, clustering in both the $x$-CoV and the $\beta$-CoV genera. Phylogenetic analysis of these new viruses has provided insight into the molecular epidemiology of CoVs, and shows that host speciation is a significant driver in CoV evolution.

**RESULTS AND DISCUSSION**

The goal of this study was to increase our knowledge of CoV diversity in bats from southern Mexico. Three sites were included in the study: Campeche, Chiapas and Mexico City (Mexico Distrito Federal; D.F.) (Fig. 1). At two of the sites (Chiapas and Campeche) bats were captured in disturbed and undisturbed habitat to investigate how anthropogenic activity may affect host and viral diversity. Such habitat gradients do not exist in D.F., which is a highly urbanized site. A total of 1046 samples were collected from 606 individuals, of 42 different bat species (Table 1).

### Host (bat) diversity

Host diversity was examined at all sites. In Chiapas, a species richness of 32 was recorded, and the calculated Shannon–Wiener diversity index ($H'$) was 2.81 (Table 2). A comparison of undisturbed and disturbed habitat in Chiapas (Shannon $t$-test) revealed no significant difference in richness and diversity ($P = 0.11$). In Campeche the overall species richness was 16 and the diversity index $H'$ was 2.167 (Table 2). Again, no significant difference in host diversity was seen between the undisturbed and disturbed habitats ($P = 0.44$). Previous work has shown that bat diversity often reflects the level of disturbance for a given habitat, with lower diversity recorded in disturbed areas (Medellín et al., 2000). No such distinction was observed here between disturbed and undisturbed sites. This may reflect the dominance of bats from the genera *Artibeus* and *Carollia* (Table 1), both of which contain species that are known to be more adaptable and resistant to the effects of habitat fragmentation (Medellín et al., 2000). An increased sampling effort including larger spatial and temporal scales will be needed to assess whether the abundance and
richness of less-well represented species alter the overall bat
diversity in each fragment. In D.F. (Mexico City), eight
species were captured and the diversity index $H'$ was 1.69
(Table 2). Sampling effort was not consistent among the
three sites, precluding any direct comparisons of diversity
between Chiapas, Campeche and D.F.

**CoV diversity**

Broadly reactive consensus PCR revealed CoV sequences in
32/606 (5.3%) bats (Table 1). Sequence analyses indicated
high phylogenetic diversity and the presence of 13 distinct
clades at the nucleotide level (Fig. 2). Clades 5a/5b and 11a/
11b had high nucleotide sequence identity and collapsed
into a single group when analysed at the amino acid level
(data not shown). Nine of the viruses clustered with known $x$-CoVs, and four clustered with $\beta$-CoVs (Fig. 2). One of
the $x$-CoVs (Mex_CoV-6) was closely related to a virus
identified previously in an *Eptesicus fuscus* bat, sampled on
the Appalachian Trail in Maryland, USA (Donaldson *et al.*,
2010). We therefore extend the known geographical range
of this virus to south-eastern Mexico and present the
discovery of a further 12 novel CoVs.

Prior to this study, very little was known about the diversity of
CoVs in the neotropics, despite the high diversity of bat
species found here (Wilson & Reeder, 2005). Here, we
demonstrate that several additional viruses from both the
genus *Alphacoronavirus* and the genus *Betacoronavirus* exist
in Mexico. This particular study was limited to the analysis of
a 329 bp fragment of the RNA-dependent RNA poly-
merase (RdRp), however, it was sufficient for the identifica-
tion of these novel strains and therefore satisfied our
primary goal of discovery.

CoV-positive sample types included 27 rectal swabs, four
oral swabs and one blood sample (annotated on Fig. 2). The
high number of positive rectal samples agrees with
previous studies, which showed CoV detection in bats to be
almost exclusively restricted to faeces (Lau *et al.*, 2005; Li
Detection in oral swabs has also been demonstrated, but
much less frequently (Carrington *et al.*, 2008).

Phylogenetic analyses of this short fragment show that
CoVs cluster based on the relatedness of host species. Fig. 2
shows that all of the $x$-CoVs detected in phyllostomid bats
cluster together; as do all $x$-CoVs discovered in mini-
opterid bats. The families Vespertilionidae and Molossidae
are closely related (Agnarsson *et al.*, 2011; Teeling *et al.,
2005), and viruses from these bats also cluster together,
though the additional presence of CoV HKU2 (from a
rhinolophid bat; Woo *et al.*, 2006) in this group is
currently unexplained. In the $\beta$-CoV genus a similar pattern is
observed. All viruses identified in rhinolophid bats cluster
together, as do viruses from the vespertilionid/molossid
group, and equally so in the related mormoopid/phylos-
tomid group. These results suggest purifying selection,
which is apparently effective at the level of host species or
genus. For example, the $x$-CoV Mex_CoV-1 was only
found in *Carollia* spp. bats, but could be present variably in
the species *Carollia sowelli* or *Carollia perspicillata*. The
same is true of the $\beta$-CoVs Mex_CoV-11a and Mex_CoV-
11b, both of which were only found in *Artibeus* spp. bats,
but which could be present in either *Artibeus lituratus* or
*Artibeus phaeotis*. Mex_CoV-6 was found in an *Eptesicus*
sp. bat and clustered very closely with the previously
identified *Eptesicus*-associated CoV (GenBank accession
no. HQ585086). Finally, the close association of Mex-CoV-
7 and -8 with *Myotis velifer* and *Tadarida brasiliensis*,
respectively, also suggest strong host specificity. These
results agree with previous studies that show individual
CoVs are associated with a single species or genus, even
among co-roosting species – including *Miniopterus*,
*Rousettus*, *Rhinolophus* and *Hipposideros* bats (Chu
*et al.*, 2006; Drexler *et al.*, 2010; Gouilh *et al.*, 2011;

Phylogenetic association of CoVs with host species/genus is
particularly evident in allopatric populations separated by
significant geographical distances; such as Mex_CoV-6 and
the previously identified HQ585086 (GenBank accession
no.) virus from Maryland, both of which were found in
*Eptesicus fuscus* and shared a very high sequence identity
despite being separated by $>2500$ km. Misra *et al.* (2009)
reported a similar observation in North America, noting
highly similar viruses from *Myotis* spp. bats in Canada and
Colorado. And the same appears to be true in *Myotis*
*ricketti* in Asia (Tang *et al.*, 2006), in *Chaerephon* and
*Rousettus* in Africa (Tong *et al.*, 2009b), and in *Nyctalus*
and *Myotis* spp. bats in Europe (August *et al.*, 2012;
Drexler *et al.*, 2010; Gloza-Rausch *et al.*, 2008). In all cases it was
concluded that even if populations of these species were
thousands of kilometres apart, highly similar CoVs could
be detected. It is important to qualify that our results are
based on a short sequence, and additional studies will be
required to assess whether our observations are consistent
when additional sequences from other genes/proteins are
considered.

Mex_CoV-5b was the only virus to be found in two distinct
(but related) genera, having been detected in both *Artibeus*
and *Carollia* bats (Fig. 2). Such findings have been reported
previously, albeit rarely (Lau *et al.*, 2012a; Osborne *et al.*, 2011;
Tong *et al.*, 2009a), and demonstrate that CoVs can
infect individuals from different genera/suborders. It is
interesting to note that this particular bat (*Carollia sowelli*,
*PMX-1232*) was captured in a disturbed habitat. Increased
efforts for viral discovery in this region will be required to
investigate whether disturbed habitats provide increased
risk or opportunity for viruses to spillover into new species,
as previously suggested (Cottontail *et al.*, 2009; Keesing
*et al.*, 2010; Suzán *et al.*, 2012). That said, the health risk to
people probably remains low, and bats should not be
viewed as a liability, especially given the vital ecosystem
functions they serve (Medellin, 2009).

Strong associations of CoVs with host species/genus could
prove to be extremely useful in identifying potential
Table 2. Evaluations of host and viral diversity at each site/habitat using the Shannon diversity index
This index takes into account the number of individuals as well as the number of taxa. A 0 value means that community has only a single taxon.

<table>
<thead>
<tr>
<th>Site</th>
<th>Habitat</th>
<th>Bat diversity</th>
<th>Viral diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>S (richness)</td>
</tr>
<tr>
<td>Chiapas</td>
<td>Undisturbed</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Disturbed</td>
<td></td>
<td>202</td>
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<tr>
<td></td>
<td>Total</td>
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<td>332</td>
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<tr>
<td>Campeche</td>
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<td></td>
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<td></td>
<td>Disturbed</td>
<td></td>
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<tr>
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<td>Total</td>
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<td>D.F.</td>
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<td>34</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>606</td>
</tr>
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</table>

**Fig. 2.** Maximum-likelihood tree of a 329 bp fragment of the RdRp from bat CoVs only (red, α-CoVs; blue, β-CoVs). All 32 positive animals from this study are presented on the tree, and begin with a PMX number that refers to the animal identity. The species of all PMX animals was confirmed by Cyt-b (cytomeglovirus b gene) barcoding. CoVs identified in this study split into 13 clades at the nucleotide level, though Mex_CoV-5a/b and Mex_CoV-11a/b collapse into single clades when assessed at the amino acid level. Each clade is indicated by a blue circle, and the total number of positive animals for each clade is indicated within. D, Disturbed habitat; UD, undisturbed habitat; U, Urban habitat. Bar, 0.05 nucleotide substitutions per site.

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Coronaviruses in bats from Mexico

Methods

Sites and sampling. Bats were captured at three different sites in Mexico, the Reserva de la Biosfera Montes Azules (Chiapas), the Reserva de la Biosfera Calakmul (Campeche), and Mexico City (D.F., Fig. 1). The first two sites, located in south-eastern Mexico represent regions of high species diversity, and are characterized by large tracts of continuous primary vegetation, while Mexico City represents a highly urbanized site. In Chiapas and Campeche bats were collected in two landscape gradients, assigned as: (1) ‘Undisturbed’ forest (UD), where any sign of human impact is largely absent; and (2) ‘Disturbed’ (D), defined as the transition zone between areas of primary vegetation and agriculture/urban, and by the presence of urban areas. Landscape units were separated by at least 10 km. Capture effort included two nights of trapping using 5 × 9 m mist nets by roosts or foraging sites. Nets were opened at dusk and remained open for 4 h consecutively. Identification of animals was made using field guides (Medellín et al., 2008). Oral and rectal swabs, and blood were collected (when possible) from each animal. For blood samples <10% of the blood volume was collected and for small bats blood was taken using protocols previously described (Smith et al., 2010). A veterinarian was present for all sampling and all animals were released safely at the site of capture. Samples were collected directly into lysis buffer and preserved at −80°C until transfer to the Center for Infection and Immunity for CoV screening. Capture and sample collection was approved by the Institutional Animal Care and Use Committee at the University of California, Davis (protocol number: 16048).

Laboratory testing. Total nucleic acid was extracted from all samples using the EasyMag (bioMérieux) platform, and cDNA synthesis performed using SuperScript III first strand synthesis supermix (Invitrogen), all according the manufacturer’s instructions. CoV discovery was performed using broadly reactive consensus PCR primers, targeting the RdRp (Quan et al., 2010). PCR products of the expected size were cloned into Strataclone PCR cloning vector and sequenced using standard M13R primers. If an individual tested positive for CoV, the species of the bat was secondarily confirmed with genetic barcoding, targeting both cytochrome oxidase subunit I and Cyt-b mitochondrial genes, as described previously (Townzen et al., 2008).

Analysis. Sequences were edited using Geneious Pro (5.6.4). Alignments were constructed using CLUSTAL W, executed through Geneious, and refined manually. Neighbour-joining and maximum-likelihood trees were built in MEGA (5.0), and bootstrapped using 1000 repetitions. Nucleotide trees that represent a consensus of both methods are presented. Evaluations of host and viral diversity at each site/habitat were made using the Shannon–Wiener diversity index (H’) using the Past 1.61 software. This index takes into account the number of individuals as well as the number of taxa. A 0 value means that the community has only a single taxon. Comparison of the Shannon–Wiener diversities (entropies) were calculated between habitat types for each region using the Shannon r-test, described by Poole (1974).
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REFERENCES


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