Identification of a Lineage D Betacoronavirus in Cave Nectar Bats (Eonycteris spelaea) in Singapore and an Overview of Lineage D Reservoir Ecology in SE Asian Bats

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Summary
Coronaviruses are a diverse group of viruses that infect mammals and birds. Bats are reservoirs for several different coronaviruses in the Alphacoronavirus and Betacoronavirus genera. They also appear to be the natural reservoir for the ancestral viruses that generated the severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus outbreaks. Here, we detected coronavirus sequences in next-generation sequence data created from Eonycteris spelaea faeces and urine. We also screened by PCR urine samples, faecal samples and rectal swabs collected from six species of bats in Singapore between 2011 and 2014, all of which were negative. The phylogenetic analysis indicates this novel strain is most closely related to lineage D Betacoronaviruses detected in a diverse range of bat species. This is the second time that coronaviruses have been detected in cave nectar bats, but the first coronavirus sequence data generated from this species. Bat species from which this group of coronaviruses has been detected are widely distributed across SE Asia, South Asia and Southern China. They overlap geographically, often share roosting sites and have been witnessed to forage on the same plant. The addition of sequence data from this group of viruses will allow us to better understand coronavirus evolution and host specificity.

Introduction
Over 70% of emerging or re-emerging infectious diseases originate in animals, with zoonotic RNA viruses responsible for the majority of these cross-species spillover events (Woolhouse et al., 2005). Zoonotic outbreaks are amplified or dampened by a number of ecological, environmental and anthropogenic factors (Karesh et al., 2012). Change in land use is a principal driver for emerging infectious diseases by modifying the wildlife–human interface (Jones et al., 2008). Increase in human populations has caused wild animals to lose their natural habitat and diminished resource availability, while bushmeat hunting and consumption have presented further opportunities for cross-species exposure (Wolfe et al., 2005). In South-East Asia, severe habitat loss has impacted the natural ecology of bats and occasionally resulted in zoonotic virus spillover, most notably in the Nipah outbreaks in Malaysia and Bangladesh (Chua et al., 2000; Hsu et al., 2004). Bats are reservoirs for many notable pathogenic viruses, including Hendra virus in Australia (Halpin et al., 2000), the recent outbreaks of Ebola virus in sub-Saharan Africa (Phillips et al., 2014).
Africa (Pourrut et al., 2009; Olival and Hayman, 2014; Ogawa et al., 2015), rabies and several other lyssaviruses (Banyard et al., 2014). Furthermore, zoonotic coronaviruses (e.g. SARS-CoV and MERS-CoV) and the human coronavirus HCoV-229E, a causal agent of the common cold, appear to have resulted from zoonotic transfer from bats, either directly or through an intermediate amplifying host (Drexler et al., 2014). Indeed, bat coronaviruses are exceptionally diverse and genetic evidence provides further support that group 1 and group 2 mammalian coronaviruses originated in bats (Vijaykrishna et al., 2007; Hu et al., 2015), highlighting the importance of bats as a potential source of new human diseases.

Coronaviruses cause a wide variety of symptoms, with human coronaviruses causing respiratory infections in humans, while other animals suffer respiratory, gastroenteric and more severe manifestations of disease (Peiris et al., 2003; Saif, 2004). Coronaviruses can be highly pathogenic, especially when they spill over into incidental hosts, as evidenced by the high fatality rates seen in the severe acute respiratory syndrome (SARS) in 2003 and the ongoing Middle East respiratory syndrome (MERS) outbreak, 9.6% and 40%, respectively (Zumla et al., 2015).

Coronaviruses are enveloped single-stranded, positive sense RNA viruses that belong to the Order Nidovirales, the Family Coronaviridae and the subfamily Coronavirinae (de Groot et al., 2012). There are four genera: Alphacoronavirus, Betacoronavirus, Deltacoronavirus and Gammacoronavirus (Adams and Carstens, 2012). Viruses of the Deltacoronavirus and Gammacoronavirus genera primarily infect birds, with avian infectious bronchitis virus a prominent member of the latter that causes systemic infections of chickens, resulting in major economic losses (Jackwood, 2012). Alphacoronaviruses infect humans (HCoV-229E and HCoV-NL63), pigs, felines, canines and a number of different bird species (Drexler et al., 2014). An alphacoronavirus, that forms a sister group to HCoV-229E, has also been recently found to commonly infect camels (Sabir et al., 2016).

Next-generation sequencing
We performed next-generation sequencing on pooled faecal material and pooled urine collected from a colony of E. spelea for virus discovery. Ten grams of faecal matter pooled from collections taken from plastic sheets laid under the colony on 14 March, 28 March and 11 April 2013 was vortexed in TBS buffer (25 mM Tris, 150 mM NaCl and Roche ULTRA protease inhibitor cocktail tablet) to resuspend the material into solution. The samples were then homogenized using silica beads (MP FastPrep – 24 bead beater; MP Biomedicals, Santa Ana, CA, USA). After homogenization, the samples underwent a series of centrifugations where the supernatant was transferred to new tubes (full protocol available upon request). After the final centrifugation step, the supernatant was discarded and the pellet was resuspended in 500 µl of TBS buffer with protease inhibitors and kept at −80°C. A library was prepared...
from the faecal processing and the serological capture extracted RNA/DNA. The SMARTer Universal Low Input RNA Kit (Clontech Laboratories, Mountain View, CA, USA) used with low concentration nucleic acids. The double-stranded cDNA library was purified with AMPure beads (Agencourt, Beckman Coulter, Brea, CA, USA), adaptors were removed with a digestion mix, and the library was amplified with an Advantage 2 PCR kit (Clontech Laboratories). The libraries were analysed on a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the reaction was run on both the Illumina HiSeq and MiSeq machines.

Urine samples from an *E. spelaea* colony in Singapore on 24 April, 8 May and 20 May 2014 were pooled and centrifuged at 10 000 g for 3 min. TRI-Reagent was added to urine viral supernatant and RNA extracted using Direct-zol™ RNA MiniPrep (#R2050; Zymo Research Corporation, Irvine, CA, USA) as per manufacturer’s instruction. RNA was subjected to in-column DNase I (#M0303S; New England BioLabs Inc., Ipswich, MA, USA) digestion as instructed in Direct-zol™ RNA MiniPrep manual. Extracted and DNase I digested RNA was treated with Ribo-Zero™ Gold rRNA Removal Kit (Epidemiology) (#MRZE706; Epicentre, Madison, WI, USA) as described in the instruction manual.

Two 500-bp next-generation sequencing cDNA libraries (urine-MiSeq-25 and urine-MiSeq-27) were constructed from the same sample using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (#E7420S; New England BioLabs Inc.) as per manufacturer’s instruction. cDNA libraries were visualized on 1.5% agarose gel, excised and purified using Zymoclean Gel DNA Recovery Kit (#D4007; Zymo Research Corporation) as instructed in Direct-zol™ RNA MiniPrep manual.

Fastq read files generated from the Illumina MiSeq and HiSeq runs were analysed using the DIAMOND alignment tool (Buchfink et al., 2015), against reference sequences in a custom protein database generated from RefSeq virus and neighbour nucleotide records (http://www.ncbi.nlm.nih.gov/genome/viruses/) (Brister et al., 2015) using a custom Perl script. DIAMOND alignment results were imported into MEGAN 6 (Huson and Mitra, 2012), using a naive LCA algorithm to perform taxonomic assignment and visualization of aligned reads. All reads taxonomically assigned to family *Coronaviridae* were extracted and pooled, and SPAdes 3.6.2 (Bankevich et al., 2012) was used to perform *de novo* assembly of pooled reads.

**PCR screening**

Oral and rectal swabs or fresh faeces from each individual were pooled and screened for coronaviruses. A total of 974 urine samples (947 samples from individuals and 27 samples pooled by collection period) collected biweekly from the *E. spelaea* colony over 4 years from May 2011 to May 2015 in addition to 150 fresh faecal pellets from the above-mentioned bat colony collected during 3 time points over 6 weeks (April–May 2014) were included. RNA was extracted from these samples using the QIAxtractor automated purification system (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and screened using coronavirus family-specific primer (PanCor IN-6 5′ – GGTTGG GACTATCCTAAGTGTGA –3′; PanCor IN-7 5′ – CCATCA TCAGATAGATCATCATA –3′) (Drosten et al., 2003) that targeted the RNA-dependent RNA polymerase gene. The PCR protocol used an initial denaturation step of 95°C for 10 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, a final extension at 72°C for 5 min. PCR products were screened on a 1.5% agarose gel and visualized on a gel doc machine. RNA from cultured Coronavirus 229E, OC43 and NL63 was extracted manually, and cDNA was synthesized to serve as positive controls.

**Phylogenetic analysis**

Coronavirus sequences representing diverse groups were downloaded from GenBank for three genes used in this study: RdRp (RNA-dependent RNA polymerase genes), *Hel* (helicase: nsp13), *E* (envelope) and *N* (nucleocapsid), including *α*-coronavirus, *β*-coronavirus (comprised of lineages A–D), and MERS-coronavirus, obtained from a wide range of hosts (bats, felines, human, avian and camels). Two Indonesian bat coronavirus that were recently detected from Moluccan naked-backed fruit bats (*Dobsonia moluccensis*) were also included (Stamatakis, 2014; Anindita et al., 2015). Partial sequences of the novel bat coronavirus generated were aligned with this data set and alignments manually edited in Geneious v9.0.3 (Biomatters, Auckland, New Zealand). Final aligned data sets comprise 82 *RdRp* sequences (2814 bp in length), 69 *Hel* sequences (1827 bp), 67 *E* sequences (240 bp) and 49 *N* sequences (578 bp) that were analysed separately (see accession numbers in Table S1). Phylogenetic trees were reconstructed using maximum likelihood implemented in RAxML v8.0.14 (Scientific Computing Group, Heidelberg Institute for Theoretical Studies, Heidelberg, Germany) (Stamatakis, 2014), and branch support was assessed with 1000 nonparametric bootstrap replicates, with only values greater than 50% indicated at the major nodes. Trees were rooted with one representative of the gammacoronaviruses (γ-CoV, accession number: AY338732).

**Host distribution mapping**

Bat species distribution and country shapefiles were downloaded from the International Union for Conservation of...
Results
The Illumina NGS data sets from libraries generated from *E. spelaea* faeces and urine produced a total of 772 coronavirus reads (Table 1). The libraries constructed from the urine samples produced 712 coronavirus reads, while the HiSeq and MiSeq runs from the faecal samples generated 60 reads. The MiSeq run on the pooled faeces produced the fewest reads. The read lengths were from a minimum of 76 bp to a maximum of 251 bp, with a mean of 240 bp (stdev 34.7 bp). A total of nine contigs were generated from the *de novo* assembly (min. 392 bp and max. 1092 bp). All 431 pooled oral–rectal samples from the six bat species were PCR-negative. The urine and faecal samples (*n* = 1124) collected from the *E. spelaea* colony were also PCR-negative.

We analysed four individual data sets (*RdRp*, *Hel*, *E* and *N*) in the phylogenetic analysis, with each data set including alpha- and beta-coronaviruses. Maximum-likelihood phylogenies show Betacoronavirus is composed of four distinct and well-supported lineages (A–D), although the intragenic relationships of beta-coronaviruses vary between the four gene trees (Figs 1 and 2). The *RdRp* phylogeny places lineage C basal to lineages A, B and D, which form a monophyletic group with only moderate support (bootstrap, BS = 58%). This interlineage relationship is consistent with a recent bat CoV study from Thailand (Wacharapluesadee et al., 2015). In contrast, in the *Hel* phylogeny, lineage A is basal to the monophyletic lineages B, C and D (BS = 88%), with lineages B and D forming a strongly supported sister group (BS = 90%). This intragenic relationship based on the *Hel* gene is also concordant with previous studies (Lau et al., 2012; Woo et al., 2012b). The *E* gene phylogeny resembles that of the *RdRp* in grouping lineages A and D as sister taxa but with low support (BS = 51%). We also compared the genetic divergence of the *RdRp* gene within the different lineages: lineage D exhibits a higher level of sequence variation with only 58.4% nucleotide identity, in contrast to lineages A (83–100% nucleotide identity), B (92–100%) and C (82–100%) that show a higher percentage similarity. The *N* gene phylogeny indicated lineage D is basal to other lineages A, B and C; however, this interlineage relationship was statistically insignificant.

The *RdRp*, *Hel*, *E* and *N* gene phylogenies all place the novel CoV identified from *E. spelaea* unequivocally in the well-supported monophyletic Betacoronavirus lineage D (*RdRp* and *Hel*: 100% BS; *E*: 91% BS; *N*: 97%). The HKU9 prototype virus for lineage D Betacoronavirus was reported from *Rousettus leschenaultii* in Hong Kong in 2005 (Woo et al., 2007). Within lineage D, partial *RdRp* sequence of the novel CoV possesses 77.2–97.4% nucleotide sequence identity with the remaining lineage D bat-CoVs. In contrast, the partial *Hel* and *E* genes of the *E. spelaea* CoV sequences exhibit greater similarity (*Hel*: 80–82.5%; *E*: 70.6–97.9%) with other lineage D viruses, although this may due to the limited number of lineage D *Hel* and *E* gene sequences that are available. The *N* gene indicated the CoV of *E. spelaea* showed more distant relationship with the remaining lineage D bat-CoVs (52–60% similarity).

The *RdRp* phylogeny indicates that the *E. spelaea* CoV is most closely related to a virus from a large Asian roundleaf bat (*Hipposideros lekaguli*), strongly supported with a bootstrap value of 100%. These two sequences form a sister group (bootstrap 93%) with coronaviruses from *Cynopterus sphinx*, *D. moluccensis*, *Hipposideros commersoni*, *Ptenochirus jagori*, *R. leschenaultii* and *Rousettus aegyptiacus* that comprise the majority of lineage D betacoronavirus sequences available.

Discussion
This is the first coronavirus detected from a bat in Singapore, and our study provides the first evidence of cave nectar bat *E. spelaea* harbouring a lineage D betacoronavirus. Coronaviruses were previously detected in *E. spelaea* in the Philippines, but no sequence data were generated (Watanabe et al., 2010). *Eonycteris spelaea* has also previously been reported to harbour a paramyxovirus in China (Yuan et al., 2014), Phnom-Penh bat virus (Queen et al., 2015), Issyk-kul virus (Calisher et al., 2006) and were also seropositive for Nipah virus in Malaysia (Yob et al., 2001).

In South-East Asia, lineage D betacoronaviruses (also referred to as Ro-BatCoV HKU9; Woo et al., 2012a,b) have been detected in eight different bat species from three bat families belonging to two suborders, Yinpterochoiroptera and Yangochiroptera: *C. sphinx*, *D. moluccensis*, *E. spelaea*, *H. lekaguli*, *P. jagori*, *R. leschenaultii*, *Scotophilus heathii* and *Scotophilus kuhlii* (Woo et al., 2007; Tsuda et al., 2012; Anindita et al., 2015). A next-generation sequencing approach in Yunnan province China detected HKU9 coronavirus sequences in a community dominated by...
Hipposideros armiger, but the host identity of the faeces tested was not confirmed (Ge et al., 2012). The bat species known to harbour lineage D Betacoronavirus are widely distributed across South Asia and South-East Asia, and there are large areas where these distributions overlap (Fig 3, Table S2). Lineage D betacoronaviruses were also detected in two bat species in Kenya, Rousettus aegyptiacus and H. commersoni, and one in Madagascar, Pteropus rufus (Tong et al., 2009; Razanajatovo et al., 2015).

Interestingly, the pooled urine samples where coronavirus was detected in the Illumina NGS were negative with traditional RT-PCR, indicating a low amount of virus. These screening results possibly indicate that there is a low prevalence in the bats in Singapore, low virus titres in the samples and/or we missed shedding foci during our sampling. The only bat species we detected coronaviruses in were the cave nectar bats (E. spelaea), and this was using deep sequencing where the majority of the reads were detected in the urine sample. We did not detect this in the other insectivorous or frugivorous bat species with this well-validated primer set; however, we did not perform NGS on samples from other species in Singapore. The majority of coronavirus detections have been from insectivorous bats, but this may have been spurred by the discovery of SARS-like CoV in Rhinolophus bat species in China (Drexler et al., 2014). Different coronaviruses can infect the same species of bats, even very divergent viruses (Wacharapluesadee et al., 2015).
The close evolutionary relationship of lineage D betacoronaviruses detected from geographically separated bat species indicates that the spread of lineage D infections in bats is not host-restricted.

In Germany, bat parturition peaks appear correlated with bat coronavirus shedding (Drexler et al., 2011); it would be beneficial to determine the pregnancy cycles of bats in the tropics, which is largely unknown or based on studies with limited geographical scope. In the tropics, the constant availability of resources means there is less dependence on temporal windows of food. In Malaysia, *E. spelaea* has been found with dependent young in 8 of 12 months, indicating that individuals are reproducing throughout the year (Kingston et al., 2006), with two peak periods of pregnancy in June and September. In the Philippines, it was also found that *E. spelaea* has two annual peaks in reproduction although with some variation (Krutzsch, 2005). The first peak centred on March or April while the second centred on or near August (Heideman and Utzurrum, 2003). These bats typically roost in large numbers in caves, although the studied colony in Singapore roosts under an overpass and numbers over 3000 individuals (Lee, unpublished data). This opens up the possibility that there is a persistent source of virus in the colonies and immunologically naive individuals have the opportunity for exposure from infected bats as they are tightly packed and may fight for specific sites in a roosting area. This is especially true for larger and older males because it is hypothesized that *E. spelaea* exhibits a resource defence polygynous mating system, where males spend significantly more time and energy for roost defence and surveillance (Bumungusi et al., 2013).

In addition, *E. spelaea*, *R. leschenaultii* and *H. armiger* co-roost in caves in India and in SE Asia and multispecies roosts may facilitate the transmission and recombination of
Fig. 3. Bat distribution map for each species in South-East Asia from which lineage D betacoronaviruses have been detected. These maps were generated from IUCN maps in QGIS. [Colour figure can be viewed at wileyonlinelibrary.com]
coronaviruses (Mendenhall, unpublished data; Struebig et al., 2005; Furey et al., 2011). Hipposideros lekaguli is thought to be associated with Rousettus species and even E. spelaea in SE Asia (Alviola et al., 2015). These species also have a large, overlapping distribution across SE Asia, which may facilitate the emergence of novel coronaviruses (Fig. 2). The foraging ecology of these bats E. spelaea is known to feed on a wide range of plant species and their high visitation frequency to the inflorescence of a few keystone plant species such as Durio zibethinus and Parkia speciosa (Heideman and Utzurrum, 2003; Krutzsch, 2005). Both C. sphinx and R. leschenaultii have also been found to feed on the same tree (Singaravelan and Marimuthu, 2004, 2008).

The discovery of this novel coronavirus demonstrates the unsampled diversity of this virus family in bats. Betacoronaviruses are responsible for two of the major coronavirus cross-species spillover events (SARS and MERS virus) from bats to incidental hosts, ultimately resulting in sustained transmission chains. The bat species where lineage D betacoronaviruses have been detected share roosting sites, do so in large numbers and high density and also forage on the same plants. These may be factors that ultimately lead to the emergence of novel virus strains (Table 2). Even though the lineage D betacoronaviruses are not implicated as viruses capable of infecting humans, understanding their evolution can help us understand the evolution and ecological aspects of this medically important virus family. Furthermore, as little is known about the evolutionary factors affecting the transmission of CoVs between hosts, additional studies, such as host cell receptor usage, are required to assess the potential risks of zoonotic transmission to humans.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1:** Accession numbers and details for coronavirus sequences used in the phylogenetic analysis.