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Research article

Newly identified viral genomes in pangolins with fatal disease

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Abstract

Epizootic pathogens pose a major threat to many wildlife species, particularly in the context of rapidly changing environments. Pangolins (order Pholidota) are highly threatened mammals, in large part due to the trade in illegal wildlife. During July to August 2018 four sick wild pangolins (three *Manis javanica* and one *Manis pentadactyla*) exhibiting a variety of clinical symptoms were rescued by the Jinhua Wildlife Protection Station in Zhejiang province, China. Although three of these animals died, fortunately one recovered after 2 weeks of symptomatic treatment. Using meta-transcriptomics combined with reverse transcription polymerase chain reaction (RT-PCR), we identified two novel RNA viruses in two of the dead pangolins. Genomic analysis revealed that these viruses were most closely related to pestiviruses and coltiviruses, although still highly genetically distinct, with more than 48 and 25 per cent sequence divergence at the amino acid level, respectively. We named these Dongyang pangolin virus (DYPV) and Lishui pangolin virus (LSPV) based on the sampling site and hosts. Although coltiviruses (LSPV) are known to be transmitted by ticks, we found no evidence of LSPV in ticks sampled close to where the pangolins were collected. In addition, although DYPV was present in nymph ticks (*Amblyomma javanense*) collected from a diseased pangolin, they were not found in the local tick population. Epidemiological investigation revealed that both novel viruses might have been imported following the illegal international trade of pangolins. Hence, these data indicate that illegal wildlife trafficking not only threatens the status of pangolin populations, but may also spread epizootic pathogens.

Key words: Pangolins; fatal disease; pestivirus; coltivirus; illegal wildlife trade.

1. Introduction

Pangolins, also known as scaly anteaters (family: Manidae, order: Pholidota), are unique among the mammals because of their overlapping scales (made of keratin) and have a geographic distribution that includes parts of Asia and Africa (Gaudin et al. 2009). Only eight pangolin species are present worldwide, four in Asia including China, and four in Africa. There is a growing interest in pangolin welfare, including the threats posed by poaching and the deforestation of their natural habitats (Hua et al. 2015). Most importantly, pangolins have become unfortunate icons for the international and domestic illegal trade in wildlife, and are the most trafficked mammal (Gaubert et al. 2018). Of the eight species, four are listed as vulnerable, two are endangered, and the remaining two are critically endangered on the International Union for Conservation of Nature Red List of Threatened Species (du Toit et al. 2017).

Pestiviruses (family: *Flaviviridae*) are enveloped RNA viruses with highly variable single-stranded positive-sense RNA genomes of ~12.3 kb that comprises a single large open reading frame (ORF) encoding a polyprotein of about 3,900 amino acids (aa) in length (Postel et al. 2015; Tautz et al. 2015). Some pestiviruses (e.g. bovine viral diarrhea virus, border disease virus, classical swine fever virus) are well-known animal pathogens that cause severe disease including contagious hemorrhagic disease in pigs and respiratory and reproductive disease in cattle and sheep (Vilcek and Nettleton 2006; Valdazo-Gonzalez et al. 2007; Moennig and Becher 2015). Wildlife disease (e.g. wild boar and deer) due to pestiviruses has also been reported (Ridpath et al. 2008; Blome et al. 2017). Following the application of molecular and genomics methods of virus discovery, a number of novel pestiviruses have been identified in recent years, including those from bats, rodents, and harbor porpoises (Wu et al. 2012; Firth et al. 2014; Smith et al. 2017; Jo et al. 2019). Finally, pestivirus-like viruses have also identified in arthropods (Shi et al. 2016a) and arthropods collected from mammals (Harvey et al. 2018). Together, these data highlight the circulation of a diverse range of pestiviruses in a broad range of animal hosts.

Coltiviruses (genus: *Coltivirus*, family: *Reoviridae*) are double-strand segmented RNA viruses. Currently, only two viruses have been defined in the genus: Colorado tick fever virus (CTFV) found in North America, and Eyach virus (EYAV) from Europe. Their RNA genome comprises twelve segments. Both viruses can cause severe disease in humans (Attoui et al. 2005; Moutailler et al. 2016), and are transmitted by ticks (Attoui et al. 2002). Recently, several novel coltiviruses have been characterized, including Tarumizu tick virus from *Haemaphysalis flava* ticks in Japan (Fujita et al. 2017), Kundal virus from *Hyalomma anatolicum* ticks in India (Yadav et al. 2019), Shelly headland virus (SHLV) from *Ixodes holocyclus* ticks in Australia (Harvey et al. 2018), and Tai Forest reovirus (TFRV) from free-tailed bats (*Chaereophon aloysiisabaudiae*) in Côte d'Ivoire (Weiss et al. 2017). Hence, there is evidently a high diversity of coltiviruses in nature and many more are likely to be identified. To date, however, it is unknown whether coltiviruses are associated with wildlife disease.

Herein, we performed a meta-transcriptomic (i.e. total RNA Sequencing) analysis of four diseased pangolins rescued by the Jinhua Wildlife Protection Station of Zhejiang province of China in 2018. From these animals, we identified and characterized the novel viral agents—a pestivirus and a coltivirus—from two pangolins. Additionally, we analyzed the clinical features and pathological changes associated with disease in these animals.

2. Materials and methods

2.1 Sick pangolins and sample collection

During July to August 2018, four sick wild pangolins were sent to the Jinhua Wildlife Rescue Station of Zhejiang province, China (Fig. 1). At the station, these pangolins received laboratory and clinical examination and subsequent treatment. Although three of the animals died, one recovered following 2 weeks of treatment. Blood and tissue samples were collected from all four animals, and ticks were collected from two (Supplementary Table S1). Pangolins and ticks were initially identified to the species level by experienced field biologists and later confirmed by analyzing sequences of the mitochondrial cytochrome b (mt-cyt b) gene or mitochondrial 16S rDNA gene as described previously (Chen et al. 2012; Guo et al. 2013).

This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the China Center for Disease Control and Prevention (CDC). All procedures for autopsy and sample collection were in strict according to the guidelines for the Laboratory Animal Use and Care from the China CDC (SYXK(Jing)2017-0021).

2.2 RNA library construction, sequencing, and data analysis

Total RNA was extracted from blood, organ tissue, and fecal samples, as well as ticks, using Nucleo Spin RNA Blood (MN, Düren, Germany), RNeasy Plus Mini Kit (Qiagen, Valencia, California USA), RNeasy Plus Universal Mini Kit (Qiagen) and TRIzol LS Reagent (Invitrogen, Carlsbad, California, USA), respectively, following the manufacturer's instructions. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). For RNA library construction, aliquots of RNA solution were pooled in equal quantity (Supplementary Table S2). The SMARTer Stranded Total RNA-Seq Kit v2 (TaKaRa, Dalian, China) and KAPA RNA HyperPrep Kit with RiboErase (HMR; KAPA, Wilmington, Massachusetts, USA) was used to construct RNA libraries from blood, organ tissue, and fecal samples, respectively. Ribosomal (r) RNA was removed using the Ribo-Zero-Gold (HMR) Kit (Illumina, San Diego, California, USA). Paired-end (150 bp) sequencing of each RNA library was performed on the HiSeqX10 platform (Illumina).

Bioinformatic analyses of the sequencing reads were undertaken as described previously (Shi et al. 2016a). In brief, adaptor- and quality-filtered sequencing reads were assembled *de novo* using the Trinity program (version 2.5.1). Viral contigs were identified by comparison (using blastn and Diamond blastx) to

Figure 1. Sampling locations (red circles) of sick pangolins from Zhejiang province, China.

the NCBI non-redundant nucleotide (nt) and protein (nr) database with *e*-values set to 1×10^{-10} and 1×10^{-4}, respectively. Likely contaminating viral sequences were excluded from the meta-transcriptomic data (Supplementary Table S2) using methods described previously (Asplund et al. 2019). In addition, the high frequency of retrovirus sequences was also excluded, as the majority of them probably were host genes, and some of them might be contaminating viral sequences, such as alpharetroviral and gammaretroviral sequences probably linked to laboratory components (Asplund et al. 2019). Finally, the putative viruses present in the blood, liver, spleen, lung, and kidney samples were confirmed by PCR. The quantity of the transcripts mapped to each viral contig was determined using the RSEM program (Li et al. 2010) implemented in Trinity.

2.3 PCR and sequencing

Total RNA was reverse transcribed using a one-step RT-PCR kit (TaKaRa). The viral RNA in ticks was detected by nested PCR targeting the conserved regions of the RNA-dependent RNA polymerase (RdRp) gene of both the pestivirus and coltivirus. To recover complete viral genomes, primers were designed based on the assembled pestivirus and coltivirus contigs obtained by meta-transcriptomics (Supplementary Table S3). The genome termini were determined by 5′/3′ RACE kits (TaKaRa).

The QIAquick Gel Extraction kit (Qiagen) was used to purify the PCR products before sequencing. Purified DNA <700 bp in length was sequenced directly, while those larger than 700 bp were first cloned into a pMD18-T vector (TaKaRa), and then transformed into JM109-143 competent cells. For each sample, at least three clones were selected for sequencing.

2.4 Phylogenetic analysis

Viral sequences were aligned using the E-INS-i algorithm implemented in MAFFT version 7 (Katoh and Standley 2013). Ambiguously aligned regions were then removed using the TrimAl program (Capella-Gutierrez et al. 2009). The best-fit model (LG+Γ) of aa sequence evolution was estimated using MEGA version 7.0 (Kumar et al. 2016). Phylogenetic trees were then estimated using the maximum likelihood (ML) method implemented in PhyML version 3.0 (Guindon et al. 2010) with bootstrap support values calculated from 1,000 replicate trees. Bootstrap values >70 per cent were considered significant. Identities among nt and aa sequences were calculated using the MegAlign program implemented in the Lasergene software package v5.0 (DNAstar).

3. Results

3.1 Illness and clinical features of sick pangolins

Between July and August in 2018, four sick wild pangolins were sent to the Jinhua Wildlife Rescue Station of Zhejiang province, China (Fig. 1). For simplicity, they were referred to here as '1-Dongyang', '2-Lishui', '3-Ruian', and '4-Wucheng', according to the location found. Morphological examination and molecular identification revealed that these pangolins belong to the Sunda pangolin, *Manis javanica* (1-Dongyang, 2-Lishui, and 4-Wucheng) and the Chinese pangolin, *Manis pentadactyla* (3-Ruian). The details and clinical signs exhibited by these pangolins are described in Table 1. All four pangolins exhibited anorexia when sent to the rescue station, and twitching and slobbering behavior were observed in 1-Dongyang and 2-Lishui. Hemorrhage and skin lesions were obvious in 1-Dongyang, whereas edema on the front legs was found in 2-Lishui. Finally, pangolin 4-Wucheng appeared febrile and contained skin lesions, whereas pangolin 3-Ruian exhibited relatively mild clinical signs.

X-ray tests revealed a large area of shadow in the left lung of pangolin 2-Lishui, suggestive of pneumonia (Supplementary Fig. S1). Due to the lack of healthy pangolin as control in this study, we used the blood biochemical values of healthy Formosan pangolins (Chin et al. 2015) as a reference. 1-Dongyang showed a significant decrease in platelet count, glucose, and cholesterol, whereas 2-Lishui exhibited a significant increase in alanine aminotransferase, aspartate aminotransferase, and amylase levels, but a decrease in total bilirubin. In addition, a lower PLT count and higher levels of alkaline phosphatases and blood urea nitrogen were observed in 4-Wucheng (Table 1 and Supplementary Table S4). Although these pangolins received careful resuscitation in the rescue station, only the pangolin 3-Ruian recovered from illness. Pangolins 1-Dongyang, 2-Lishui, and 4-Wucheng died on 3, 16, and 5 days after the admission, respectively.

3.2 Pathologic changes in dead pangolins

Congestion was observed in the liver and lung of pangolin 1-Dongyang following autopsy, although no obvious pathological changes were observed in other inner organs. Consistent

Table 1. Background information, clinical features, and examination of the rescued pangolins.

Pangolin ID	Species	Gender/age	State	Clinical features	Clinical examination	
					X-ray	Hematological analysis
1-Dongyang	*M. javanica*	Male/adult	Dead	Anorexia, twitch, slobber, hemorrhage,[a] and skin lesions[b]	Normal	PLT ↓, GLU ↓, cholesterol ↓, and amylase ↑
2-Lishui	*M. javanica*	Female/adult	Dead	Anorexia, twitch, slobber, and edema[c]	Large area of shadow on the left lung	ALT ↑, AST ↑, amylase ↑, and total bilirubin ↓
3-Ruian	*M. pentadactyla*	Female/juvenile	Alive	Anorexia, anxiety, and edema[c]	–	–
4-Wucheng	*M. javanica*	Female/adult	Dead	Anorexia, fever, and skin lesions[b]	Stones in the stomach	PLT ↓, ALP ↑, BUN ↑

[a]On the left ear.
[b]On the face and paws.
[c]On the front legs.
PLT, platelet count; GLU, glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; ALP, alkaline phosphatase.

with the obvious congestion, histological tests revealed hemorrhage in the liver and lung (Supplementary Fig. S2). In addition, pyknotic nucleus of the hepatic cells, lymphocyte pyknosis in spleen, necrosis of the hepatic plate, and glomerular necrosis were observed. Finally, collagen fiber degeneration was observed in the liver, spleen, and lung (Supplementary Fig. S2).

For pangolin 2-Lishui, obvious necrosis was observed in the lung, kidney, and spleen, and congestion was observed in the liver (Supplementary Fig. S2) in addition to mesenteric lymphadenopathy. Notably, many milky white lesions were present in both lungs, especially in the left lower lobe (1×2 cm). Histological tests revealed necrosis in the liver, spleen, lung, kidney, trachea, and small intestinal, as well as hemorrhage in the lung (Supplementary Fig. S2).

3.3 Identification of viral agents by meta-transcriptomics and PCR

To identify the possible etiologic agents of disease in the four pangolins, eight meta-transcriptomic libraries from blood, liver, spleen, lung, kidney, and fecal samples were constructed, generating a total of 306,908,179 paired-end sequence reads. *De novo* assembly revealed the high abundance of a pestivirus- and coltivirus-like virus in all the meta-transcriptomic libraries of the pangolin 1-Dongyang and 2-Lishui, representing 6–80 and 1–29 per cent of total viral contigs, respectively (Supplementary Table S2). Notably, despite the presence of other putative viruses (Supplementary Table S2), only the pestivirus- and coltivirus-like virus could be successfully identified by PCR. As those putative viral sequences could not be confirmed by PCR and probably were from contamination; hence, they were not possible etiologic agents of disease in four pangolins. Additional assays revealed that the novel pestivirus was only present in 1-Dongyang, whereas the novel coltivirus was only present in 2-Lishui. No potential bacterial and fungal pathogens were found in the libraries generated from 1-Dongyang and 2-Lishui. Finally, no abundant viral, bacterial and fungal sequences were found in the libraries generated from blood and tissue samples of 3-Ruian and 4-Wucheng (Supplementary Table S2).

Genetic analysis revealed that the novel pestivirus shared <57 per cent nt similarity to known pestiviruses, while the novel coltivirus showed <68 per cent nt similarity to known coltiviruses. Considering that they are related, yet clearly genetically distinct, from known members of *Pestivirus* and *Coltivirus* (see below), we designated these two newly identified viruses as Dongyang pangolin virus (DYPV) and Lishui pangolin virus (LSPV), respectively, reflecting their hosts species and the geographic location of sampling. Attempts at virus isolation by cell culture (BHK-21, Vero-E6, and DH82) and inoculation of suckling mice proved unsuccessful.

To determine the distribution of the newly identified viruses, each organ was tested by PCR. Consequently, DYPV was identified in the heart, liver, spleen, lung, kidney, brain, blood, throat swab, and fecal sampled from pangolin 1-Dongyang, as well as the nymph ticks (*Amblyomma javanense*) collected from this animal. Interestingly, the virus was not identified in the adult ticks also sampled from 1-Dongyang. Similarly, LSPV was also identified in a broad range of tissue organs including heart, liver, spleen, lung, kidney, blood, throat swab, and fecal matter of 2-Lishui.

3.4 Genetic features of DYPV and LSPV

To further characterize both viruses, primers were designed based on the sequences obtained here and those of related viruses described previously. In this manner we were able to recover the complete genomes of DYPV from 1-Dongyang and the ticks (*A. javanense*) collected from this animal. The genetic features of DYPV are described in Fig. 2a. Notably, viruses obtained from the pangolin and ticks were closely related to each other, although still exhibited almost 2 per cent nt sequence difference. In addition, both viruses were genetically and phylogenetically distinct from known viruses of the genus *Pestivirus*, with >43 per cent nt and >48 per cent aa differences (Supplementary Table S5). As with classical pestiviruses, the genome of DYPV encodes twelve proteins. All known cleavage sites of the NS3 protease were observed in DYPV (Supplementary Fig. S3). In addition, the NS4A-NS4B cleavage site, located at Position L_{2426}/A_{2427} in BVDV1 and L_{2336}/A_{2337} in CSFV, appeared at Position L_{2315}/K_{2316} site in DYPV (Supplementary Fig.S3).

Notably, despite the use of both meta-transcriptomic and PCR methods, only nine genome segments (1–5 and 8–11) were obtained from the novel coltivirus—in animal 2-Lishui—and our attempt to recover segments 6, 7, and 12 failed. Details of the genetic features of LSPV are described in Fig. 2b. All recovered segments had the consensus sequences (GAG/AUU/A) at the 5′-terminus and (G/CAGUC) and the 3′-terminus (Fig. 3a), respectively. However, the genome sequences of LSPV were distinct from those of recognized coltiviruses, with <75 per cent aa similarity (Supplementary Table S6). Like other coltiviruses, LSPV Segment 9 also contained two ORFs. However, the sequences adjacent to the UGA stop codon, which are the signal of readthrough, were different from known coltiviruses. Notably, however, a similar pattern was observed in SHLV (Fig. 3b), the closest relative of LSPV, that was sampled from ticks in Australia.

3.4 Phylogenetic relationships of the newly identified viruses

To determine the evolutionary relationships among the newly identified and previously recognized viruses, we estimated ML phylogenetic trees based on aa sequences (Figs 4 and 5).

In phylogenies based on the aa sequences of the entire coding sequences and the NS3 genes, DYPV formed a highly distinct lineage, confirming that it represents a novel pestivirus. Notably, the DYPV lineage occupied the basal position within the Artiodactylous lineage, with those pestiviruses present in rodents and bats forming more divergent lineages. In the phylogenies based on the aa sequences of the RdRp gene and putative RNA methyltransferase gene (VP2; Fig. 5), LSPV also formed a distinct lineage within the genus *Coltivirus*. Interestingly, however, the virus was most closely related to Shelly headland identified in ticks (*I. holocyclus*) sampled from the long-nosed bandicoot (a marsupial) in Australia (Harvey et al. 2018).

3.5 Phylogenetic analysis of pangolin sequences

The Sunda pangolin (*M. javanica*) is geographically distributed widely in South-East Asia, including Indonesia (Java, Sumatra, Borneo, and the Lesser Sunda Islands), Malaysia, Singapore, The Philippines, Thailand, Vietnam, Laos, and Cambodia (Zhang et al. 2015), whereas Chinese pangolins (*M. pentadactyla*) are relatively commonplace in Southern China (Choo et al. 2016).

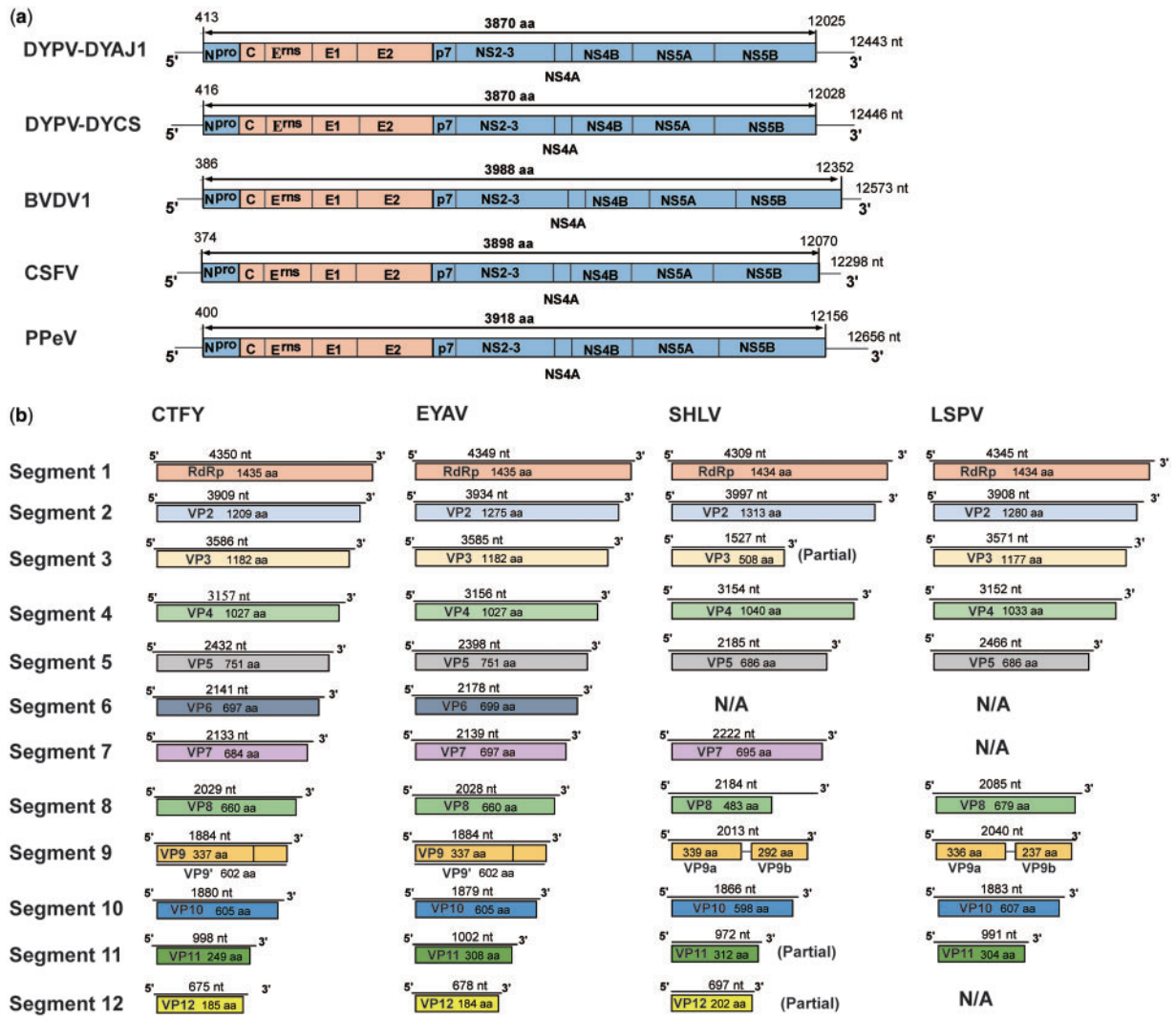

Figure 2. Schematic of the annotated DYPV and LSPV genomes. (a) Genome comparison between DYPV and known pestiviruses. (b) Genome comparison between LSPV and known coltiviruses.

To determine the likely geographic origin of these sick pangolins, sequences of mt-*cyt* b gene were amplified from their tissue. Genetic analysis revealed that all the sequences obtained from Sunda pangolins in this study fell into the *M. javanica* group, while the Chinese pangolins clustered with those of *M. pentadactyla*. Notably, three Sunda pangolins sampled here were very closely related to those from Indonesia, Malaysia, and Thailand and Singapore, with 99.9, 99.7, and 99.9 per cent nt sequence identity, respectively, indicating that they were most likely (illegally) imported into China from abroad. In contrast, the pangolin 3-Ruian (*M. pentadactyla*) clustered together with those from Taiwan/China, with 99.1 per cent nt sequence identity, suggesting that this animal was not imported. As no sequences related to the *A. javanense* mitochondrial 16S rDNA were available, we could not determine the origin of the *A. javanense* ticks collected from sick Sunda pangolins. Hence, a systemic effort should be considered to establish comprehensive databases for the speciation of arthropod vectors and as a tool for determining the geographic origin of the collected arthropods.

3.6 Molecular investigation of DYPV and LSPV in local ticks

As DYPV was identified in ticks (*A. javanense*) collected from pangolin 1-Dongyang, and LSPV was closely related to SHLV also identified in ticks (*I. holocyclus*) from Australia, we collected ticks at the locations from where the sick pangolins were found. Consequently, 452 ticks representing 7 species were collected, including 220 *Haemaphysalis hystricis*, 147 *Rhipicephalus microplus*, 49 *Haemaphysalis longicornis*, 11 *Ixodes granulatus*, 11 *Rhipicephalus haemaphysaloides*, 9 *Rhipicephalus sanguineus*, and 5 *Haemaphysalis mageshimaensis*. Unfortunately, neither DYPV nor LSPV were identified in these ticks by meta-transcriptomics and nested RT-PCR.

4. Discussion

Pangolins are insectivorous, predominantly nocturnal, and predate almost exclusively on ants and termites, with a strong preference for particular insect species (Lin et al. 2015;

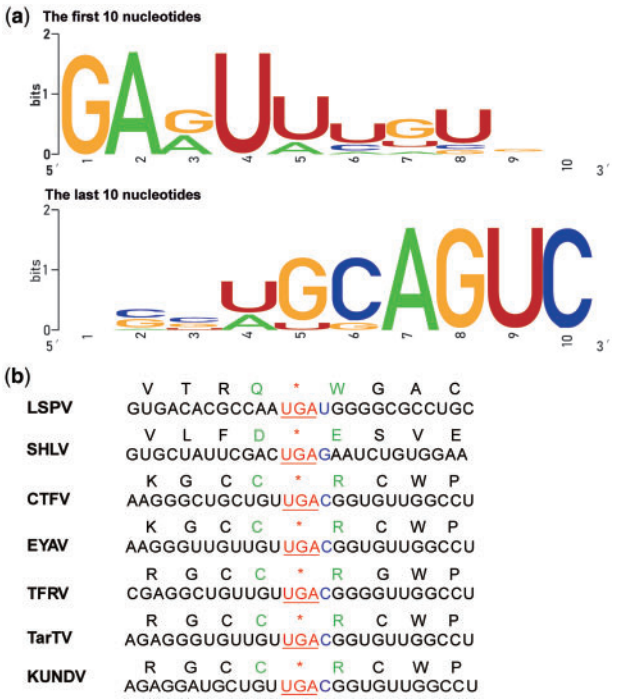

Figure 3. Characteristics of the LSPV genome. (a) Sequence conservation of the 5′- and 3′-terminal 10 nts in genomic segments of the LSPV genome were analyzed and visualized using Weblogo. (b) Sequence information around the stop codon of segment VP9 of LSPV and known coltivirus.

Choo et al. 2016). Eight species of pangolins are present in Africa and Asia, with habitat loss and changes in their living environment seriously affecting their population status. Most importantly, pangolins have been greatly impacted by the illegal international and domestic wildlife trade for traditional medicine or meat (Zhang et al. 2015). Hence, pangolins are listed as vulnerable, endangered or critically endangered on the IUCN Red List of Threatened Species (du Toit et al. 2017). Notably, pangolins may also be threatened by epizootic pathogens, either present in their original habitats or in their new environments following translocation. Indeed, the translocation or trafficking of domestic and wild animals plays an important role in the spread of many epizootic pathogens (Lin et al. 2012; Kosmider et al. 2013; Peeler et al. 2015).

In this study, four pangolins—three *M. javanica* likely from Indonesia, Malaysia, and Thailand, and one *M. pentadactyla* probably of local origin—were found to be suffering disease and were sent to a local rescue station for treatment. Unfortunately, three animals died, whereas one recovered. To date, reports on pangolin disease have been rare and mainly limited to those caused by bacteria and parasites (Mohapatra et al. 2016; Jabin et al. 2019). There is no available literature on viral infections of pangolins until the recent identification of Parainfluenza Virus 5, sendai virus, and coronavirus from Sunda pangolins in China (Liu et al. 2019; Wang et al. 2019). Using a combination of meta-transcriptomic and PCR methods, we identified two novel RNA viruses—a pestivirus and a coltivirus—in two dead pangolins. Based on their clinical signs (Table 1), all these four pangolins appeared to suffer infectious disease. However, as no complete clinical data and laboratory parameters were obtained from these animals, we could not clearly define the disease they suffered.

Pestiviruses are well-known animal pathogens that cause significant economic loss, infecting both domestic (e.g. pigs, cattle, sheep, and goats) and wild (e.g. wild boars and ruminants) animals (Vilcek and Nettleton 2006). Pestivirus infections may be subclinical or cause a range of clinical signs including acute diarrhea, acute hemorrhagic syndrome, acute fatal disease, as well as a wasting disease. Herein, a novel pestivirus, designated DYPV, was identified in multiple organs in one of the sick pangolins [1-Dongyang (*M. javanica*)] that had clear pathological changes. Phylogenetic analysis indicates that it represents a novel member of the genus *Pestivirus*. Although more detailed confirmatory results are required, these data suggest that DYPV-like pestiviruses may be responsible for the hemorrhagic disease observed in the pangolins.

To date, it is commonly believed that vertebrates, rather than invertebrates, are the main hosts of pestiviruses (Smith et al. 2017). Recently, however, a novel pestivirus, named Fairfax Lookout virus, was identified in ticks (*Ixodes trichosuri*). However, given its phylogenetic position next to mammalian pestiviruses, as well as its the extremely low abundance, it was proposed that the virus was associated with the vertebrate host rather than from the tick itself (Harvey et al. 2018). In this study, DYPV was not only identified in the pangolins, but also in nymph ticks collected from the pangolins. However, DYPV was not found in the adult ticks also sampled from pangolin 1-Dongyang. Interestingly, although DYPV from ticks was closely related to that sampled from pangolins, there were ~2 per cent nt differences across the viral genome suggesting that they are separated by multiple transmission events. Phylogenetic analysis indicated that Sunda pangolins, including the pangolin 1-Dongyang, were most likely imported into China from Indonesia, Malaysia, and the Philippines (Fig. 6). In addition, *A. javanense* ticks have not found in Zhejiang province (Chen et al. 2010), DYPV was not identified in locally collected ticks, and we did not observe this virus in previous large scale tick sampling studies (Li et al. 2015; Shi et al. 2016a,b, 2018). Together, these observations suggest that the virus was probably imported from abroad with the illegal trafficked pangolin.

Coltiviruses are well-known tick-borne pathogens that can cause human disease. For example, CTFV causes mild febrile illness or more severe disease including infection of the central nervous system, and/or hemorrhagic fever (Goodpasture et al. 1978; Attoui et al. 2005). Additionally, EYAV infections have been associated with human neurological disease (Moutailler et al. 2016). Notably, although some coltiviruses (e.g. CTFV, EYAV, TFRV) have been detected in wildlife (such as bats and rodents), there is no clear evidence that these viruses can cause disease in animals (Moutailler et al. 2016; Weiss et al. 2017; Williamson et al. 2019). In this study, a novel coltivirus, named LSPV, was identified in one of the sick pangolins [2-Lishui (*M. javanica*)]. Given the high abundance of LSPV in the meta-transcriptomic data, combined with the clinical features and pathologic changes appeared in the pangolin (2-Lishui), as well as the detection of LSPV in several organs, our data suggest that LSPV may have caused systemic infection and the death of the pangolin in question, although this will need to be confirmed with additional data.

It is generally recognized that the coltivirus genome comprises twelve segments of linear double-stranded RNA (Fujita et al. 2017). Recently, a novel coltivirus, Forest reovirus (TFRV), was identified in free-tailed bats (*C. aloysiisabaudiae*; Weiss et al. 2017). Interestingly, the virus genome lacks segments 6, 7, and 12, and it was proposed that these missing segments were not

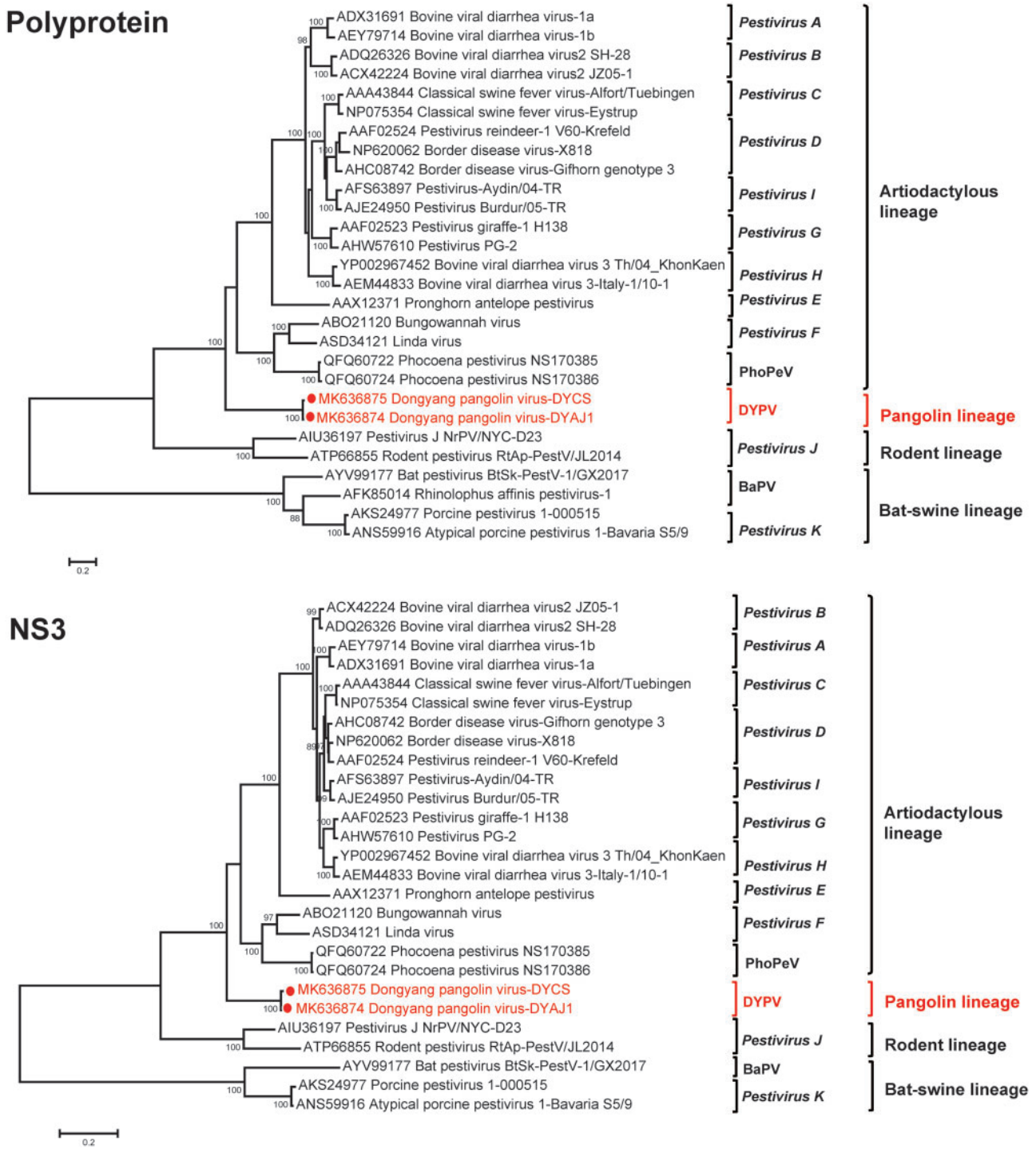

Figure 4. ML trees based on aa sequences of the entire coding sequences (polyprotein) and NS3 genes of DYPV and other known pestiviruses. The numbers at nodes indicate bootstrap support values after 1,000 replications. Bootstrap values higher than 70 per cent were considered significant and shown on the trees.

identified probably due to low similarities between TFRV and known coltiviruses (Weiss et al. 2017). Strikingly, as with TFRV, we were unable to identify viral segments 6, 7, and 12 in LSPV, despite the use of methods that previously obtained the complete genome of the highly divergent Jingmen tick virus (Qin et al. 2014). It is therefore clear that additional studies are needed to infer whether these segments are indeed absent from LSPV and TFRV. Finally, as pangolin 2-Lishui may have been imported from Indonesia (Fig. 6) it is possible that LSPV, like DYPV, was also imported into China.

Finally, despite some discussion concerning the role of pangolins in the emergence of the novel coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2;Wahba et al. 2020; Wong et al. 2020), the cause of the corona virus disease 2019 (COVID-19) outbreak (Wu et al. 2020; Zhou et al. 2020), we have not found coronaviruses in these pangolins by meta-transcriptomic

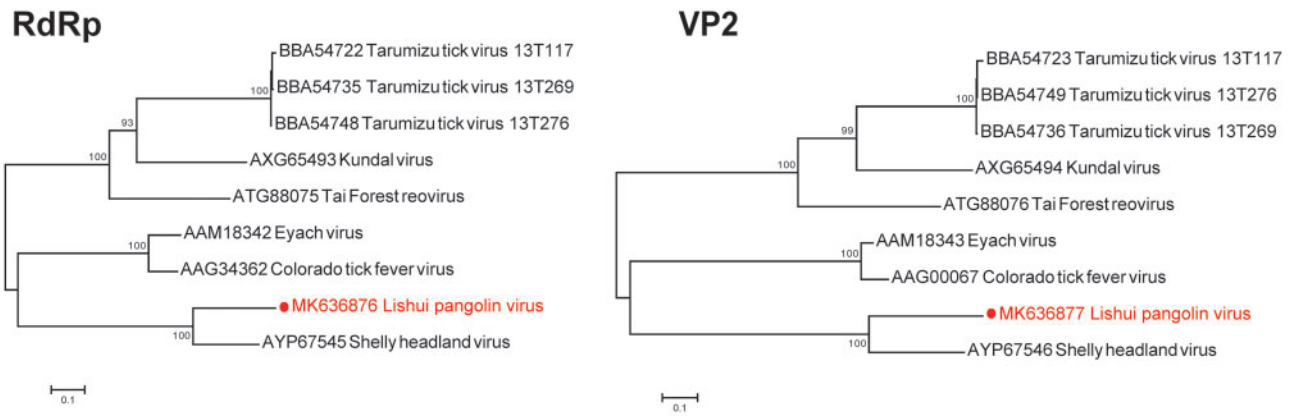

Figure 5. ML trees based on aa sequences of the RdRp genes and putative RNA methyltransferase (VP2) genes of LSPV and other known coltiviruses. The numbers at nodes indicate bootstrap support values after 1,000 replications. Bootstrap values higher than 70 per cent were considered significant and shown on the trees.

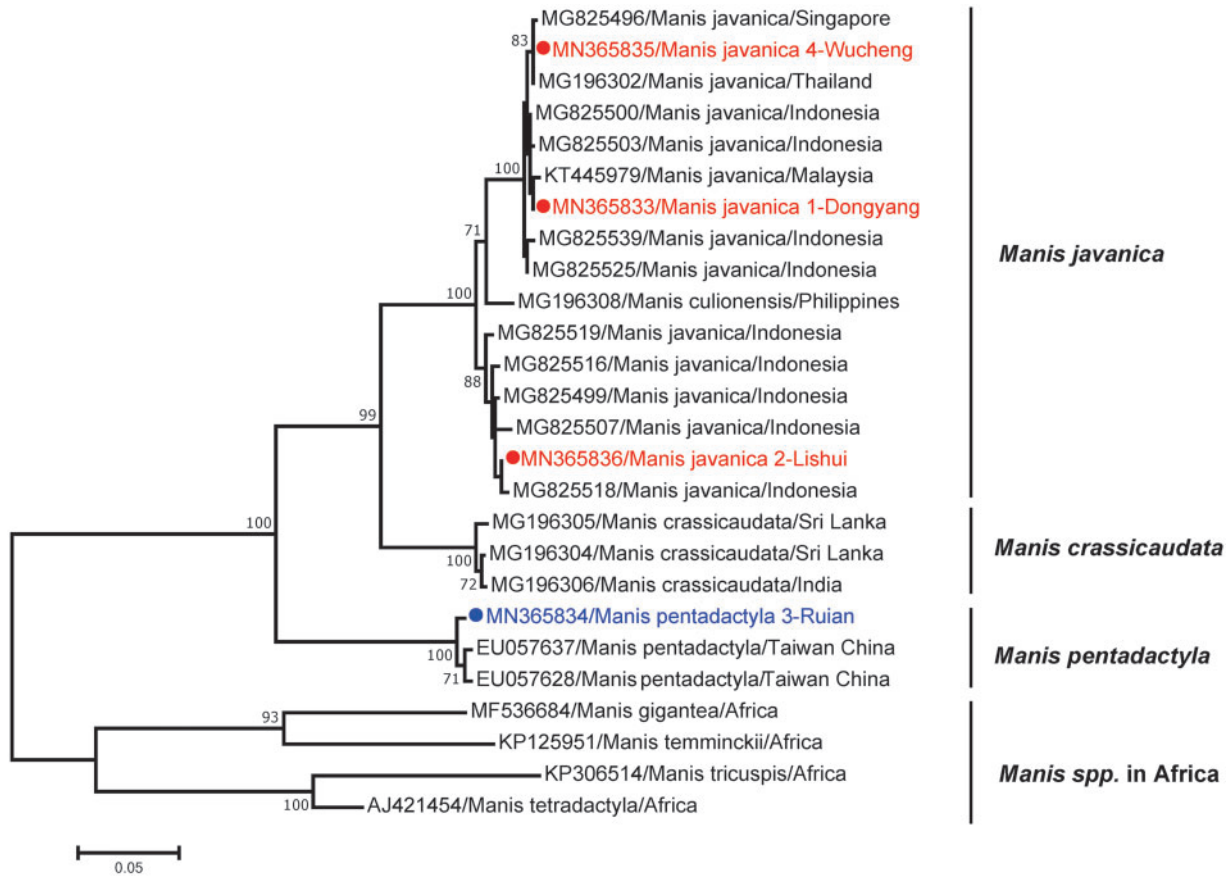

Figure 6. ML tree based on nt sequences of the mt-cyt b gene of four pangolins and other known pangolins. The numbers at nodes indicate bootstrap support values after 1,000 replications. Bootstrap values higher than 70 per cent were considered significant and shown in the trees.

and PCR methods. Therefore, more efforts are needed to infer the role of pangolins in the transmission of SARS-CoV-2.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

Data Availability

All viral genome sequences, 16S rDNA and mt-cyt b gene sequences generated in this study have been deposited in GenBank under the accession numbers MK636874–MK636884, MN365828–MN365832, and MN365833–MN365836.

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