Molecular detection of a novel paramyxovirus in fruit bats from Indonesia

Michihito Sasaki1, Agus Setiyono3†, Ekowati Handharyani3†, Ibenu Rahmadani4, Siswatiana Taha5, Sri Adiani6, Mawar Subangkit3, Hirofumi Sawa1, Ichiro Nakamura2 and Takashi Kimura1*

Abstract
Background: Fruit bats are known to harbor zoonotic paramyxoviruses including Nipah, Hendra, and Menangle viruses. The aim of this study was to detect the presence of paramyxovirus RNA in fruit bats from Indonesia.

Methods: RNA samples were obtained from the spleens of 110 fruit bats collected from four locations in Indonesia. All samples were screened by semi-nested broad spectrum reverse transcription PCR targeting the paramyxovirus polymerase (L) genes.

Results: Semi-nested reverse transcription PCR detected five previously unidentified paramyxoviruses from six fruit bats. Phylogenetic analysis showed that these virus sequences were related to henipavirus or rubulavirus.

Conclusions: This study indicates the presence of novel paramyxoviruses among fruit bat populations in Indonesia.

Background
The genus Henipavirus in the subfamily Paramyxovirinae, family Paramyxoviridae, contains two highly pathogenic viruses, i.e., Hendra virus and Nipah virus. Hendra virus causes fatal pneumonia and encephalitis in horses and humans. The first case was identified in 1994 and Hendra virus disease still continues to arise sporadically in Australia [1,2]. Nipah virus also causes acute encephalitis and respiratory symptoms in animals and humans, with a high mortality rate. Outbreaks of Nipah virus have occurred in Malaysia, Singapore, Bangladesh, and India [1,2]. Henipaviruses have been isolated from fruit bats including Pteropus vampyrus [3], Pteropus hypomelanus [4], Pteropus lylei [5], Pteropus poliocephalus, and Pteropus alecto [6], which are considered to be their natural reservoirs. Epidemiological studies demonstrate that Hendra and/or Nipah virus-seropositive fruit bats are widely distributed throughout Asian countries [7-11]. No human cases of henipavirus infection have been reported in Indonesia, although Pteropus vampyrus that are seropositive for both Nipah virus and Hendra virus are distributed nationwide [12,13]. These findings indicate the presence of henipavirus or henipa-like viruses in Indonesian fruit bats, suggesting the need for further epidemiological investigations.

Menangle virus, belonging to the genus Rubulavirus of the Paramyxoviridae family, has been identified in pteropus bats from Australia [14]. Menangle virus is a zoonotic paramyxovirus that causes febrile illness with rash in humans [15]. Tioman virus, belonging to the genus Rubulavirus, has also been isolated from Pteropus hypomelanus on the island of Tioman, Malaysia [16]. Although Tioman virus showed antigenic cross-reactivity to Menangle virus, the pathogenicity of Tioman virus remains unclear. There have been no reports of rubulavirus infections in the Indonesian fruit bat population.

The current study used molecular sequencing and phylogenetic analyses to identify RNA sequence from potential paramyxoviruses in fruit bats from Indonesia.

Results
A total of 110 fruit bats belonging to four different species were sampled from four locations in Indonesia (Figure 1). Pteropus vampyrus was captured in Panjalu District (n = 26) and Lima Puluh Kota District (n = 20). Other pteropus bats captured in Popayato District (n = 4) and Paguyaman District (n = 25) were considered to be closely related to Pteropus hypomelanus, based on the shared nucleotide sequence identity of their 16S rRNA (96%) and...
cytochrome b (cyt b) (95%) with corresponding sequences from Pteropus hypomelanus (GenBank/EMBL/DDBJ entry AF069537 and AB062472). Acerodon celebensis was captured in Paguyaman District (n = 18). Dobsonia bats that were captured in Paguyaman District (n = 17) had high sequence similarity with 16S rRNA (96%) and cyt b (94%) from Dobsonia moluccensis (JN398196 and FJ218484). Information on the samples is summarized in Table 1.

RNA samples from each fruit bat spleen were screened using semi-nested broad spectrum reverse transcription PCR (RT-PCR), as described previously [17]. The primers were designed based on a conserved sequence within the RNA polymerase large (L) gene of the Paramyxovirinae subfamily, which includes Avulavirus, Rubulavirus, Respirovirus, Morbillivirus, and Henipavirus [17]. Semi-nested RT-PCR was positive for 1/26 (4%) Pteropus vampyrus specimens captured in Panjalu District. The size of PCR product detected in the positive sample (sample number IFBPV01/2010) was 584 bp, and the amplified viral sequence excluding the primer-derived sequences (530 bp) was deposited in GenBank (accession number AB691542). Positive results with amplification of the 530 bp viral sequence (excluding the primer-derived sequences) were also obtained for 4/25 (16%) Pteropus sp. captured in Paguyaman District, i.e., IFBPV25/2011 (AB691543), IFBPV32/2011 (AB691544), IFBPV39/2011 (AB691545), and IFBPV46/2011 (AB691546), and for 1/18 (6%) Acerodon celebensis specimens captured in Paguyaman District, i.e., IFBPV32/2012 (AB710472). No positive results were obtained for the 20 Pteropus vampyrus captured in Lima Puluh Kota District, the four Pteropus sp. captured in Popayato District, or the 17 Dobsonia sp. captured in Paguyaman District (Table 1).

BLAST search showed that all six amplicons shared less than 65% nucleotide identity with homologous fragments of paramyxovirus sequences previously deposited in GenBank. Deduced pairwise amino acid identities were then calculated to compare the homologous region with known paramyxovirus L proteins (Table 2). IFBPV32/2011 shared 98% nucleotide identity and 100% amino acid identity with IFBPV39/2011, suggesting that they belonged to the same strain. IFBPV01/2010, IFBPV32/2011, IFBPV39/2011, and IFBPV46/2011 were most closely related to Nipah virus of all the known paramyxoviruses. IFBPV25/2011 shared

| Species          | Location collected       | Year collected | Nested RT-PCR |
|------------------|--------------------------|----------------|---------------|
| *Pteropus vampyrus* | Panjalu district         | 2010           | 26            | 1             |
| *Pteropus vampyrus* | Lima Puluh Kota district | 2011           | 20            | 0             |
| *Pteropus sp.* | Popayato district         | 2011           | 4             | 0             |
| *Pteropus sp.* | Paguyaman district        | 2011           | 23            | 4             |
| *Pteropus sp.* | Paguyaman district        | 2012           | 2             | 0             |
| *Acerodon celebensis* | Paguyaman district       | 2012           | 18            | 1             |
| *Dobsonia sp.* | Paguyaman district        | 2012           | 17            | 0             |

*Genetically closely related to Pteropus hypomelanus.
**Genetically closely related to Dobsonia moluccensis.
Table 2 Pairwise amino acid identities of predicted L gene products compared with known paramyxoviruses

| Genus       | Species                        | IFBPV01/2010 | IFBPV25/2011 | IFBPV32/2011, IFBPV39/2011 | IFBPV46/2011 | IFBPV32/2012 |
|-------------|--------------------------------|--------------|--------------|----------------------------|--------------|--------------|
| IFBPV01/2010| 37                             | 90           | 66           | 35                         |              |              |
| IFBPV25/2011| 90                             | 36           | 65           | 38                         |              |              |
| IFBPV32/2011, IFBPV39/2011 | 66   | 38           | 65           | 38                         |              |              |
| IFBPV46/2011| 35                             | 70           | 36           | 38                         |              |              |
| Avulavirus  | Avian paramyxovirus 3           | 29           | 30           | 34                         | 35           | 35           |
|             | Newcastle disease virus         | 34           | 34           | 36                         | 40           | 40           |
| Rubulavirus | Mapuera virus                   | 41           | 40           | 40                         | 64           |              |
|             | Menangle virus                  | 34           | 33           | 37                         | 77           |              |
|             | Mumps virus                     | 38           | 36           | 40                         | 68           |              |
|             | Tioman virus                    | 34           | 34           | 37                         | 78           |              |
|             | Tuhoko virus 2                  | 38           | 36           | 38                         | 72           |              |
| Respirovirus| Human parainfluenza virus 1     | 51           | 49           | 52                         | 34           |              |
|             | Human parainfluenza virus 3     | 51           | 51           | 52                         | 35           |              |
|             | Sendai virus                    | 51           | 49           | 51                         | 34           |              |
| Morbillivirus| Canine distemper virus          | 57           | 56           | 55                         | 34           |              |
|             | Measles virus                   | 58           | 57           | 56                         | 37           |              |
|             | Rinderpest virus                | 57           | 57           | 56                         | 38           |              |
| Henipavirus | Hendra Virus                    | 67           | 66           | 69                         | 35           |              |
|             | Nipah Virus                     | 70           | 69           | 70                         | 35           |              |
| Unclassified| J-virus                        | 61           | 62           | 60                         | 38           |              |

72% amino acid sequence identity with Tuhoko virus 2, which was isolated from Rousettus leschenaulti in China [18]. IFBPV32/2012 shared 78% amino acid sequence identity with Tioman virus.

A phylogenetic analysis was performed based on the deduced amino acid sequences (176 amino acids) from the six nucleotide sequences obtained (Figure 2). The phylogenetic tree showed that IFBPV01/2010, IFBPV32/2011, IFBPV39/2011, and IFBPV46/2011 formed three distinct branches that were closely related to the genus Henipavirus. IFBPV25/2011 and IFBPV32/2012 were most closely related to the genus Rubulavirus (Additional file 2).

The amino acid sequence GDNQ is highly conserved in the viral RNA polymerase of non-segmented negative-stranded RNA viruses and it is responsible for polymerase activity [19, 20]. However, this motif is replaced by GDNE in the L protein of Henipavirus [20, 21]. The region encoding the GDNQ/GDNE motif was amplified by RT-PCR to determine whether the putative henipavirus-like nucleotide sequences contained the characteristic GDNE motif in the L protein. The deduced amino acid sequence comparison showed that only IFBPV46/2011 encoded the GDNE motif, among the six samples obtained (Figure 3).

Virus isolation was attempted using African green monkey kidney (Vero) and rabbit kidney (RK13) cells because these cell lines are used for the isolation or propagation of various paramyxoviruses [3, 4, 6, 16, 22]. After serial passages, an RT-PCR assay detected no paramyxovirus RNA in the culture supernatants (data not shown).
Discussion

Four henipavirus-like and two rubulavirus-like nucleic acid sequences were detected in fruit bats from Indonesia. The phylogenetic analysis showed that these novel viral sequences possessed considerable sequence divergence, suggesting that a variety of paramyxoviruses are circulating in the Indonesian fruit bat population.

In addition to fruit bats of the genus *Pteropus*, partial paramyxovirus sequences were identified from fruit bats of the genus *Eidolon, Rousettus* and *Epomophorus* [18, 23, 24].
To our knowledge, this is the first study that has detected paramyxovirus RNA from fruit bats of the genus Acerodon. This finding broadens the number of megabat genera which are associated with paramyxoviruses.

Tuhoko virus, Tioman virus, Menangle virus, and Mapuera virus have been identified as fruit bat-associated rubulaviruses. Menangle virus causes central nervous system degeneration in pigs and it also infects humans [14,15]. Therefore, it would be useful to investigate infections of humans or domestic animals with the novel rubula-like viruses detected in this study.

Conclusions
This study identified unique paramyxovirus sequence from three species of fruit bats (Pteropus vampyrus, Pteropus hypomelanus and Acerodon celebensis), potentially representing three new henipaviruses and two new rubulaviruses. To the best of our knowledge, this is the first study to identify viral genome sequence from potential paramyxoviruses in the tissues of fruit bats from Indonesia. Local people consume bat meat in Indonesia, so further epidemiological and experimental studies are needed to determine the risk of fruit bat-associated paramyxovirus infection of humans in Indonesia.

Methods
Animal samples and RNA extraction
Fruit bats were captured in: Panjalu District, West Java Province during February, 2010 (n = 26); Lima Puluh Kota District, West Sumatra Province during February, 2011 (n = 20); Popayato District, Gorontalo Province during February, 2011 (n = 4); and Paguyaman District, Gorontalo Province during February, 2011 and February, 2012 (n = 23 and n = 37, respectively). All animal research was performed in accordance with the ethical guidelines of the Animal Care and Use Committee of Veterinary Teaching Hospital, Bogor Agricultural University. All of the spleen tissues from fruit bats were divided into two samples. Samples for RNA extraction were stored in RNAlater (Life Technologies, Carlsbad, CA), followed by RNA extraction with TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Samples for virus isolation were frozen at -80°C. These samples were exported with TRIzol reagent (Life Technologies). The inner primer duplex PCR was used in the semi-nested PCR was designed for a nucleotide sequence encoding the GDNQ/GDNE motif from each sample was amplified using a SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies). The inner primer PAR-F2 used in the semi-nested PCR was designed for a nucleotide sequence encoding this GDNQ/GDNE motif.

Viral isolation
Virus isolation was attempted in the biosafety level (BSL)-3 facility at the Research Center for Zoonosis Control, Hokkaido University. Frozen spleen tissues were homogenized (10%, wt/vol) in MEM containing penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml) (all obtained from Life Technologies). 

Phylogenetic analysis
The obtained nucleotide sequences and the deduced amino acid sequences were compared with those of known paramyxoviruses. Nucleotide and amino acid identity values were calculated using GENETYX software ver. 10 (GENETYX, Tokyo, Japan). Multiple sequence alignments were constructed based on the amino acid sequences deduced from the six nucleotide sequences obtained using the MEGA5 program [27]. Phylogenetic analysis was performed using the neighbor-joining method with 1000 bootstrap replicates [28,29].
Technologies). The homogenates were clarified by centrifugation at 1000 x g for 5 min and inoculated onto Vero cells and RK13 cells for 2 h at 37°C. Cells were washed with phosphate-buffered saline (–) and cultured with MEM containing 2% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). All cells were subcultured every 5 or 6 days. After three serial passages, RNA samples were prepared from each culture supernatant using a High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) and analyzed by semi-nested RT-PCR, as described above.

Accession number
The amplified nucleotide sequences reported in this study have been deposited in the GenBank nucleotide database under accession numbers AB691542 to AB691546, AB748559 to AB748561 and AB710472. AB691543 and AB710472 are composed of three overlapping sequences that were amplified using three primer sets; PAR-F2 and PAR-R, AVU-RUB F2 and AVU-RUB R, and the primers for the amplification of GDNQ/GDNE motif.

The GenBank/EMBL/DDBJ accession numbers of the amino acid sequences used in this study were ACB46872 (Avian paramyxovirus 3), NP_071471 (Newcastle disease virus), NP_054714 (Mumps virus), YP_001249278 (Avian paramyxovirus 3), NP_071471 (Newcastle disease virus), NP_067153 (Human parainfluenza virus 3), AAA75501 (Sendai virus), NP_604442 (Human parainfluenza virus 1), NP_056797 (Rabies virus), NP_473843 (Australian bat lyssavirus), NP_066251 (Ebola virus), and ACT79225 (Marburg virus).

Additional files

| Additional file 1: Phylogenetic analysis of amino acid sequences derived from partial L gene fragments. |
| Additional file 2: Phylogenetic analysis of amino acid sequences derived from partial L gene fragments. |

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
M. Sasaki, A. Setiyono, E. Handharyani, and T. Kimura designed research; M. Sasaki, A. Setiyono, E. Handharyani, S. Adiani, I. Rahmadani, S. Taha, M. Subangkit, I. Nakamura, and T. Kimura performed research; M. Sasaki, A. Setiyono, E. Handharyani, H. Sawa and T. Kimura analyzed the data; M. Sasaki, H. Sawa and T. Kimura wrote the manuscript. All authors read and approved the final manuscript.

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