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"Preliminary investigations on the ecology and the phylogenetic relationship of coronaviruses of Sri Lankan bats."

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University of Hong Kong <u>and</u>

Department of Zoology
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xii. Publications/Communications arising from the project during the reporting period: In preparation

Impact of research results:

i. Relevance of results achieved to scientific advancement

With the pan-coronavirus and pan-astrovirus RT-PCR assays, two coronavirus positives and 14 astrovirus positives were detected from the samples. Two main groups of astroviruses were recorded from Sri Lankan bats. The astroviruses recorded from all microchiropteran bats in Sri Lanka clustered together with those of the same genus recorded from different regions world, to form monophyletic groups. In contrast, the astrovirus recorded from *R. leschenaulti* from Wavulgalge differed from any of the astroviruses recorded from all other microchiropteran bats recorded anywhere else in the world. This astrovirus clustered among the astrovirus recorded from other major groups of mammals such as dolphins, sealions and cheetah and showed a unique relationship to typical human astroviruses. Moreover, no astrovirus from a bat species relating to any other group of mammal has previously been recorded. These findings are likely to provide new insights into evolution of astroviruses and reinforce the role of bats as a reservoir of viruses with potential to pose a zoonotic threat to human health.

ii. Relevance of results achieved to national/socio-economic development

This is the first record of the presence of viruses in a Sri Lankan bat population. Results of this study shows there is a high prevalence of viruses of Sri Lankan bats. Our findings of a novel astrovirus in the fruit bat *Rousettus leschenaulti* relating to those recorded in humans found within a relatively small geographic area, highlight the need for expanding the study to include other species of bats on a more broader scale to arrive at

iii. Dissemination/application of research output

A manuscript based on this study is being prepared.

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Tables and Figures

Summary

Bats are increasingly recognized to harbor a wide range of viruses and in most instances these viruses appear to establish long term persistence in these animals. Amongst the 60 viral species reported to be associated with bats, 59 are RNA viruses, which are potentially important in the generation of emerging and re-emerging infections in humans. They are the reservoir of a number of human zoonotic diseases including Nipah, Ebola and SARS. Severe acute respiratory syndrome (SARS) has been described by the World Health Organization (WHO) as the first, serious and readily transmissible disease to emerge in the 21st century which caused over 770 deaths in seven months.

The cause of SARS has been conclusively identified as a previously unknown coronavirus. Masked palm civets have been identified as the intermediate host while the definitive hosts may be the horseshoe bats (genus *Rhinolophus*).

Until now, all the studies on bat coronaviruses have been confined to Hong Kong and Mainland China. In Sri Lanka over 1/3 of mammalian species are bats, most of which roost in human dwellings or in close contact with humans. Therefore we initiated a study to determine the species and the phylogenetic relationships of coronaviruses and astroviruses by genetic analysis of several species of Sri Lankan bats. We also wanted to see any possible relationship between ectoparasites of bats and coronaviruses.

During the study period, 10 species of bats from 12 locations were screened. This included 7 species of microchiropetans (*Rhinolophus rouxii, Hipposideros lankadiva, H. speoris, H. ater, M. schreibersii, Megadema spasma, Taphozous melanopogon)* and 3 megachiropteran species (*Pteropus giganteus, Cynopterus sphinx, Rousettus leschenaulti)*. The roosting sites of bats included 3 caves, one mine, four buildings, two open roosts of Flying foxes and two mist nettings. Bats were also screened for the presence of ectoparasites.

During the study, 367 individuals representing all species of bats (except *P. giganteus*,) were screened for parasites. Parasites were recorded from *R. leschenaulti*, *M. spasma*, *H. speoris*, *H. ater*, *R. rouxii* and *M. schreibersii*. No parasites were recorded for *T. melanopogon*, *H. lankadiva* and *C. sphinx*. The ectoparasites recorded were from seven families; Nycteribiidae (Bat Flies),

Streblidae (Bat Flies), Trombiculidae (Mites), Spinturnicidae (Mites), Ixoididae (Ticks), Argasidae (Ticks) and Ischnopsyllidae (Fleas). Nycteribiidae and Spinturnicidae were the most abundant families from which, bat parasites were recorded

With the pan-coronavirus and pan-astrovirus RT-PCR assays, two coronavirus positives and 14 astrovirus positives were detected from the samples. Coronaviruses were found only from *M.* schreibersii captured from Wavulgalge. The astroviruses were found from *R. rouxii, H. ater, H. speoris, M. spasma, M. schreibersii, H. lankadiva* and *R. leschenaulti.* Viruses were not recorded at all from *C. sphinx, P. giganteus* and *Taphozous melanopogon.* We also did not find any significant association between the presence of parasites and presence of viruses.

The astroviruses recorded from Sri Lankan bats separate into two main groups. All microchiropterans into one and those recorded from the fruit eating bat, *R. leschenaulti* into the other. The astrovirus recorded from *R. leschenaulti* from Wavulgalge (SL99 F- Figure 3.7) differed from the astroviruses recorded from all other microchiropteran bats. This astrovirus is clustered among the astrovirus recorded from other major groups of mammals such as dolphins, sealions and cheetah and showed a unique relationship to typical human astroviruses. Moreover, no astrovirus from a bat species relating to any other group of mammal has previously been recorded. These findings are likely to provide new insights into evolution of astroviruses and reinforce the role of bats as a reservoir of viruses with potential to pose a zoonotic threat to human health.

This is the first record of the presence of viruses in a Sri Lankan bat population. Our findings of novel coronaviruses and astroviruses in bats in Sri Lanka provide information on the ecology of these viruses in bats in this country. When integrated with similar studies of bats in other contries, these results would also provide a wider picture on the ecology of the viruses in nature. The remarkably high prevalence and presence of novel astrovirus relating to those recorded in humans found within a relatively small geographic area highlight the need for study in other species of bats and in a more broader scale.

1. Introduction

Severe acute respiratory syndrome (SARS) has been described by the World Health Organization (WHO) as the first, serious and readily transmissible disease to emerge in the 21st century. The epidemic first appeared in Southern China in late 2002 (Guan *et al.*, 2003) and was finally contained in July 2003 after spreading to 29 countries worldwide (WHO, 2004). It is estimated that over 8,000 people were infected, with 774 reported deaths (WHO, 2003). The last known cases occurred in April 2004 after a laboratory acquired infection in China. The global response to the SARS epidemic, coordinated by the WHO, led to the rapid identification of the causal agent, the development of diagnostic tests for the virus, the initiation of treatment protocols, estimation of key epidemiological factors affecting the spread of the disease and the implementation of a range of public health interventions (WHO, 2003, WHO, 2004; Anderson *et al.*, 2005).

The cause of SARS has been conclusively identified as a previously unknown coronavirus (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003: Peiris *et al.*. 2003). Early reports suggested a wild animal reservoir for the virus and attention focused on the wildlife trade in Southern China (Xu *et al.*. 2004). Numerous animal reservoirs of the SARS coronavirus have since been identified (Shi & Hu, 2007). Masked palm civets (*Paguma larvata*) have been most consistently identified as the intermediate host responsible for passing the virus to humans (Guan *et al.*, 2003: Song *et al.*, 2005; Wang *et al.*, 2005), while the definitive hosts may be the horseshoe bats (genus *Rhinolophus*) (Wang *et al.*, 2006).

The origin of the etiological agent of the SARS coronavirus (SARS-CoV), remains to be elusive. However, it is reported that bats are natural hosts of coronaviruses closely related to those responsible for the SARS outbreak. These viruses, termed SARS-like coronaviruses (SL-CoVs), display greater genetic variation than SARS-CoV isolated from humans or from civets. The human and civet isolates of SARS-CoV nestle phylogenetically within the spectrum of SL-CoVs, indicating that the virus responsible for the SARS outbreak was a member of this coronavirus group.

Recent studies have suggested bats as a source of emerging infections in humans (Wong *et al.*, 2007). Amongst the 60 viral species reported to be associated with bats, 59 are RNA viruses, which are potentially important in the generation of emerging and re-emerging infections in humans (Wong *et al.*, 2007). The prime examples of these are the Lyssaviruses and Henipavirus. The transmission of Nipah, Hendra and perhaps SARS coronavirus and Ebola virus to humans, may involve intermediate amplification hosts such as pigs, horses, civets and primates, respectively. Several instances of emerging diseases in humans appear to be caused by the spill over of viruses endemic to bats, either directly or through other animal intermediaries (Mistra *et al.*, 2009). These researchers detected RNA of a group 1 coronavirus from *Myotis lucifugus* and DNA from an as-yet undescribed polyomavirus from female *M. lucifugus* and *M. californicus.*

Understanding the natural reservoir or introductory host, the amplifying host, the epidemic centre and at-risk human populations, are crucial in the control of emerging zoonosis. The association between bat coronaviruses and certain lyssaviruses with the particular bat species, implies co-evolution between specific viruses and bat hosts. Cross-infection between the huge number of bat species may generate new viruses which are able to jump the trans-mammalian species barrier more efficiently. Certain families of bats including the *Pteropodidae, Molossidae, Phyllostomidae*, and *Vespertilionidae* are most frequently associated with known human pathogens. Therefore, a systematic survey of bats is warranted to better understand the ecology of these viruses.

Since identifying coronavirus as the causative agent of SARS (Peiris *et al*, 2003), an intensive effort has been undertaken to identify the animal reservoir of the precursor of the SARS virus. This search led to the discovery of a number of novel coronaviruses in different species of bats (Poon *et al.*, 2005, Chu *et al.*, 2006, Tang *et al.* 2006, Woo *et al.* 2006, Woo *et al.*, 2007). The diversity of these viruses suggests that in evolutionary terms, bats were the original reservoir from which all human and other mammalian coronavirus lineages have emerged (Vijaykrishna *et al.*, 2007). The precursor of the SARS virus was also identified in *Rhinolophus* species of bats (Lau, *et al.*, 2005, Li *et al.*, 2005). This discovery has lead to a new horizon in our understanding of the diversity of coronaviruses in nature. The classification of coronaviruses is also likely to change as a result.

Until now, all the studies on bat coronaviruses have been confined to Hong Kong and Mainland China. Relatively little surveillance of coronaviruses in bats in other parts of the world has been reported and none from South Asia. In the light of the discovery mentioned above, and the migratory habits of bats, we undertook a preliminary surveillance of coronaviruses in several species of bats in Sri Lanka. Screening of coronaviruses of bats is important since several species of bats in Sri Lanka (*Rhinolophus rouxii, Hipposideros speoris, Megaderma lyra, Megaderma spasma,* and some vespertilionids) roost in human dwellings and are in close contact with humans. In fact, recent studies have shown that most bat colonies and humans share the same dwellings (Yapa, *et al.*, 2000). Further, large colonies of flying foxes are located in populated areas, specially botanical gardens and parks (e.g. Vihara Maha Devi Park, Colombo; Peradeniya Botanical Gardens).

It was also deemed necessary to examine whether a relationship exists between bat ectoparasites and presence of coronaviruses and also the ecology of these coronaviruses.

The objectives of this project are to:

I. Investigate the presence of coronaviruses in selected species of bats in Sri Lanka.
II. Determine the species and the phylogenetic relationships of coronaviruses, by genetic analysis.
III. Determine the ecology of these coronaviruses
IV. Determine whether a relationship exists between ectoparasites of bats and coronaviruses.

In order to study the ecology of these viruses and to learn the possible mechanism of cross transmission that takes place among different species, we selected colonies of bats representing:

I. Species differentiation (bats from different families)
II. Variation in roosting habitat (roosting in the open, caves and buildings)
III. Species composition (roosts with sympatric species and roosts with single species).
IV. Variation in population sizes (from < 50 individuals to several thousands)

2. Materials and Methods

2.1. Site Selection

Sites for sampling bats were selected from the descriptions based on a previous study (Yapa *et al*, 2000) and other known locations based on personal experience. Several criteria were used in selection of sites for this study viz. (a) Species composition (single species and multi-species assemblages and sympatric species) (b) roost types (caves, buildings, open roosts) and (c) colony size (small, medium, large). In addition, mist netting was carried out in the premises of the University of Colombo and in a home garden in Jaela.

2.2. Capturing bats

The roosts were visited during day time when all bats have returned after foraging. Bats were captured inside the roosts, often using hand nets. Whenever feasible, entrances were blocked using mist nests to prevent bats from escaping the roosts. This not only helped us to capture individuals who attempted to leave the roosts, but also effectively prevented bats from being exposed to attacks by diurnal predators (birds of prey).

Each captured bat was transferred to individual, clean cotton bags and kept in a dark, cool and quiet place for subsequent examination. Species were identified using the checklist given by Bates & Harrison (1997). The gender was differentiated by examining the genital area of each individual. The maturity was determined by using a combination of several criteria, viz. tooth wear, fur colour, appearance of the wing. Size difference (length of the forearm), translucent appearance of the wing and changes in the patterns and rates of closure of the cartilaginous epiphyseal growth plates were used to estimate the age of juvenile bats. In its simplest form, this method is used to qualitatively distinguish between young bats and adults. By trans-illuminating the wing of an individual using a headlight (or holding the wing against the bright sky), it was possible to visualize the cartilaginous zone of the long phalanges because less mineralized tissue, allows more light to pass through and thus appears lighter than bone.

Bats were kept in captivity for the shortest possible time and immediately released inside the roost after sample collection. If free flying bats were trapped using mist nests, they were released at the site of capture. Particular attention was paid to minimize the stress to bats during capture and handling, as well as to avoid disturbance inside the cave and near the colony and to preserve the surroundings as first encountered.

2.3. Collection of Ectoparasites

Once the rectal and throat swabs were taken, each bat was screened for parasites. The ecto-parasites were collected using the method described by Whitaker, 1988. The captured bats (one at-a-time) were temporarily transferred to a cloth sack and a piece of cotton wool soaked with ether was introduced to the sack containing the bat. This procedure, weakly anesthetized both bats as well as the parasites. Thereafter, bats were taken out and the entire body surface (fur, wing and tail membrane, ears, head, neck, feet) was thoroughly examined for presence of parasites. Large fleas were directly picked off the host using a fine pair of forceps or a small paint brush. The mites that were attached to the body surface were collected with an arrow pointer. Thereafter, the entire body surface of bats was brushed onto a white paper with a fine bristled tooth brush. In some instances, a drop of coconut oil was placed on top of the mite to loosen it from the skin and then removed as described above. All collected parasites were stored in labelled glass vials containing 70% of Ethyl Alcohol, to be identified later.

2.4. Sample collection and RNA extraction

Rectal swabs and throat swabs were collected from the wild caught bats using sterile Ultrafine Aluminium Applicator Swabs (Fisher brand, Calcium Alginate Fibre Tipped, Fisher Heath Care, made in USA). Both, a rectal swab and a throat swab, were taken from each captured bat. To take a specimen, the swab was gently inserted into the anus (for a rectal swab) and into the buccal cavity (for throat swabs), while the bat was being securely held. To get a good specimen, the swab was slowly rotated (inside the mouth or the anus) while moving back and forth and then gently withdrawn after a few seconds. The lower part containing the smear of the swab was then cut and placed in a RNA stabilization medium RNAlater (QIAgen, Germany) to limit RNA degradation.. For each smear, a fresh swab was used. Each sample was marked by a code (species, gender, age, roost, sample number, smear type) and was temporarily stored in a cool box and transported to the laboratory of the Department of Zoology, University of Colombo. The specimens were stored at -70^0C in viral transport medium (VTM) and shipped on dry ice to the University of Hong Kong where they were analyzed at the laboratory of Prof. J.S.M. Peiris (Chair Professor, Department of Microbiology, The University of Hong Kong and Research Director, HKU-Pasteur Research Centre, Hong Kong.), by RT-PCR and virus culture, for coronaviruses. Viral transport medium (VTM) was prepared by dissolving into 1 liter of Medium 199 (Sigma-Aldrich) with the following antimicrobial drugs and chemicals: 12.5 g of penicillin G sodium salt, 0.05 g of ofloxacin, 0.1 g of nystatin, 2 g of polymycin B sulfate salt, 2.5 g gentamicin, 1 g sulfamethoazole, 0.2 g sodium hydroxide and 2.2 g sodium hydrogen carbonate. The solution obtained was adjusted to pH 7 – 7.5 by adding 4% sodium hydrogen carbonate solution. The VTM prepared was sterilized by filtering through a 0.22 um filter.

Identification of parasites was carried out at the Department of Zoology, University of Colombo using the keys provided by Whitaker, 1988).

Upon arrival in Hong Kong, the samples were processed immediately and the original samples were kept at -80°C for long term storage. Nucleic acid was extracted from the samples using QiagenQIAamp viral RNA mini kit following the protocol provided by the manufacturer. RNA extracted was subjected to RT-PCR assays for the detection of coronaviruses. Since astroviruses are important causes of diarrhoea in many animal species including humans and has a potential to pose a threat to human health (Zhu et al., 2009) although the main objective of the study was to screen samples for coronaviruses, as an extension, we also looked for the presence of astroviruses by astroviruses specific RT-PCR. The positive specimens were subjected to subsequent genetic sequence amplification and sequencing to derive full length viral genomes.

2.5. Viral Detection

RT-PCR for coronaviruses, virus culture and DNA sequencing RNA eluted was reverse transcripted to produce first-strand cDNA using SuperScript III reverse transcription kit (Invitrogen). The cDNA synthesized was tested by PCR using coronavirus conserved primers. PCR products obtained were sequenced. Characterization of the coronaviruses detected was done based on the genetic sequence obtained.

For details of methodology, please refer to the publication by Poon *et al.* 2005 (attached hereto).

Three batches of samples (n1 = 77, n2= 200, n3= 90; total 367) were sent to University of Hong Kong for analysis.

3. Results

3.1. Sampling Sites and bat species

During the study period, 10 species of bats from 12 locations were screened. This included 7 species of microchiropetans and 3 megachiropteran species (Table 3.1). The roosting sites of bats included 3 caves, one mine, four buildings, two open roosts of Flying foxes and two mist nettings. Wavulgalge, Fashiengala cave and Kurunegala House were sympatric roosts whereas the rest of the colonies were single-species colonies (Table 3.1). *Rhinolophus rouxii* was captured from two locations, one from a sympatric roost (Wavulgalge cave) and the other, in a building Muththettuwegama house (single species colony). *Hipposideros speoris* was recorded in 3 roosts (Wavulgalge, Fashiengala Cave and Kurunegala house). *Rousettus leschenaulti* was recorded in 3 locations (Naugala Cave, Wavulgalge and at National Meuseum.) All other species [*Hipposideros lankadiva* (from Bogala Mine), *Hipposideros ater* (from Kurunegala House), *Taphozous melanopogon* (from Fasheingala Cave) , *Miniopterus schreibersii* (from Wavulgalge), *Megaderma spasma* (Galapatha Temple)] were recorded only in single locations. *Cynopterus sphinx* was mist-netted at two locations, at University of Colombo and in a home garden in Jaela. Fecal samples of *Petropus giganteus* were collected from two open roosts, one from Vihara Maha Devi Park in Colombo and the other located at Hunupitiya.

The colony sizes of bats varied, between species as well as roost types. The largest colony was recorded in Wavulgalge for *R. leschenaulti* exceeding 50,000 individuals. In contrast, the colonies (of the same species) located in Naugala cave and the National Museum were comparatively small numbering, <1000 bats. Similarly, although, a large population of *R. rouxii* was recorded in Wavulgalge (>10,000), population size in Muththetuwegama house was relatively small (about 200 individuals). The populations of

H. speoris and *M. schreibersii* recorded in Wavulgalge were <200 bats. The colony size about 400 individuals of *H. lankadiva* was recorded from Bogala mine. The canopy roosts of *P. gignateus* in Hunupitiya, Jaela and Vihramadevi Park were comparatively large exceeding 1000 individuals. Small populations (<50 bats) were recorded for *H. ater* (in Kurunegala house) and *M. spasma* (in Galapatha temple).

3.2. Study of Ectoparasites

During the study, 367 individuals representing all species of bats (except *P. giganteus*, which was not captured) were screened for parasites. The species of host bats, the number of bats screened and the parasites recorded are given in the Table 3.2. Parasites were recorded from *R. leschenaulti*, *M. spasma*, *H. speoris*, *H. ater*, *R. rouxii* and *M. schreibersii*. No parasites were recorded for *T. melanopogon*, *H. lankadiva* and *C. sphinx*.

Table 3.1. Details of the species composition, colony size and the roost parameters of the bat colonies sampled during the study. (* Mist netting sites; roost parameters not known; ** - not captured)

Roost	Type	Roosting pattern	Species	Colony size
Wavulgalge	Cave	Sympatric	*R. leschenaulti*	50,000-60,000
			M. schreibersii	<200
			R. rouxii	10000-12000
			H. speoris	<100
Fa-Shiengala	Cave	Sympatric	*H. speoris*	<100
			T. melanopogon	<100
Naugala cave	Cave	Single	*R. leschenaulti*	~1000
Bogala Mine	Mine	Single	*H. lankadiva*	~400
Kurunegala house	Building	Sympatric	*H. speoris*	<50
			H. ater	<50
Galapatha Temple	Building	Single	*M. spasma*	25-30
Muththetuwegama	Building	Single	*R. rouxii*	<200
National Museum	Building	Single	*R. leschenaulti*	~200
Vihara Mahadevi park	Open/Tree	Single	*P. giganteus***	~ 3000
Hunupitiya	Open/Tree	Single	*P. giganteus***	~1000
Univ. of Colombo	*	*	*C. sphinx*	*
Jaela	*	*	*C. sphinx*	*

The ectoparasites recorded were from seven families; Nycteribiidae (Bat Flies), Streblidae (Bat Flies), Trombiculidae (Mites), Spinturnicidae (Mites), Ixoididae (Ticks), Argasidae (Ticks) and Ischnopsyllidae (Fleas). (Table 3.2).

Nycteribiidae and Spinturnicidae were the most abundant families from which, bat parasites were recorded. From the total number of parasites recorded, 41% were Nycteribids whereas 28% were from the family Spinturnicidae (Figure 3.1). 18% of parasites were from the Family Argasidae whereas Streblids accounted for 10%. Bat flies from the families Nycteribiidae and Streblidae were the mostly wide spread parasites recorded from the bats species screened.

Nycteribiids were recorded from 33 individuals of bats whereas Streblids were recorded from 17 individuals. There were four species of Nycteribids, (*Eucampsipoda latisterna, Stylidia phillipsi, Nycteribia allotopa* and *Penuicillida indica). E. latisterna* was the most abundant bat fly recorded for this family. 73% of Nycteribids were from this species followed by *S. phillipsi* (13%), *P. Indica* (11%) and *N. allotopa* (3%).

Table 3. 2: Family, species and numbers of parasites recorded from each of the host bat species at different roosting sites.

Rousettus leshenanlti, Naugala cave

	Details of the parasite		
Host identity	**Family**	**Species**	**# of parasites**
NG/Rl/1	Nycteribiidae	*Eucampsipoda latisterna*	5
	Spinturnicidae	*Ancystropus sp1*	6
	Spinturnicidae	*Onchocelus kanheri*	5
NG/Rl/2	Nycteribiidae	*Eucampsipoda latisterna*	2
NG/Rl/3	Nycteribiidae	*Eucampsipoda latisterna*	4
NG/Rl/4	Nycteribiidae	*Eucampsipoda latisterna*	4
	Streblidae	*Megastrebla sp1*	2
	Spinturnicidae	*Ancystropus sp1*	18
	Ischnopsyllidae	*Thaumapsylla breviceps*	3
NG/Rl/5	Nycteribiidae	*Eucampsipoda latisterna*	8
	Streblidae	*Megastrebla sp1*	1
NG/Rl/6	Nycteribiidae	*Eucampsipoda latisterna*	5
	Argasidae	*Ornithodoros sp* larval form	3
NG/Rl/7	Nycteribiidae	*Eucampsipoda latisterna*	3
	Argasidae	*Ornithodoros sp* larval form	3
	Spinturnicidae	*Ancystropus sp1*	3
NG/Rl/8	Nycteribiidae	*Eucampsipoda latisterna*	3
NG/Rl/9	Nycteribiidae	*Eucampsipoda latisterna*	3
NG/Rl/10	Nycteribiidae	*Eucampsipoda latisterna*	3
	Spinturnicidae	*Ancystropus sp1*	1
	Unidentified mite		2

***Rhinolophus rouxi* - Muththettuwegama**

	Details of the Parasite		
Host identity	**Family**	**Species**	**# of parasites**
MG/Rr/1	Nycteribiidae	*Stylidia phillipsi*	4
MG/Rr/2	Nycteribiidae	*Stylidia phillipsi*	4
	Streblidae	*Brachytarsina sp 1*	1
MG/Rr/3	Streblidae	*Brachytarsina sp 1*	2
MG/Rr/4	Nycteribiidae	*Stylidia phillipsi*	2
	Streblidae	*Brachytarsina sp 1*	2
MG/Rr/8	Streblidae	*Brachytarsina sp 1*	2
MG/Rr/9	Streblidae	*Brachytarsina sp 1*	2
	Unidentified Intestinal nematode		12
MG/Rr/10	Trombiculidae	Unidentified	1

Table 3.2: contd…

Rhinolophus rouxii **- Wavulgalge**			
Host identity	**Details of the parasite**		
	Family	**Species**	**# of parasites**
WC/Rr/4	Spinturnicidae	Unidentified	4
WC/Rr/5	Spinturnicidae	*Paraperighichus sp 1*	2
WC/Rr/6	Ixoididae	Haemaphysalis larval form	1
	Nycteribiidae	*Stylidia phillipsi*	1
WC/Rr/7	Nycteribiidae	*Stylidia phillipsi*	1
WC/Rr/9	Spinturnicidae	*Paraperighichus sp 1*	1
WC/Rr/10	Spinturnicidae	*Paraperighichus sp 1*	1

Rousettus leschenaulti **- Wavulgalge**			
Host Identificatity	**Details of the «parasite**		
	Family	**Species**	**# of Parasites**
WC/Rl/1	Nycteribiidae	*Eucampsipoda latisterna*	3
	Streblidae	*Raymondia pagodarum*	1
WC/Rl/2	Nycteribiidae	*Eucampsipoda latisterna*	4
	Argasidae	*Ornithodoros sp larval form*	7
	Spinturnicidae	*Ancystropus sp1*	3
WC/Rl/3	Nycteribiidae	*Eucampsipoda latisterna*	1
	Spinturnicidae	*Ancystropus sp1*	3
WC/Rl/4	Nycteribiidae	*Eucampsipoda latisterna*	3
	Spinturnicidae	*Ancystropus sp1*	2
WC/Rl/5	Nycteribiidae	*Eucampsipoda latisterna*	1
	Argasidae	*Ornithodoros sp larval form*	7
WC/Rl/6	Nycteribiidae	*Eucampsipoda latisterna*	4
	Argasidae	*Ornithodoros sp larval form*	1
	Spinturnicidae	*Ancystropus sp1*	3
	Ischnopsyllidae	*Thaumapsylla breviceps*	1
WC/Rl/7	Nycteribiidae	*Eucampsipoda latisterna*	2
	Argasidae	*Ornithodoros sp larval form*	3
	Spinturnicidae	*Ancystropus sp1*	2
WC/Rl/8	Nycteribiidae	*Eucampsipoda latisterna*	7
	Spinturnicidae	*Ancystropus sp1*	3
WC/Rl/9	Nycteribiidae	*Eucampsipoda latisterna*	1
	Argasidae	*Ornithodoros sp larval form*	12
	Spinturnicidae	*Ancystropus sp1*	3
WC/Rl/10	Argasidae	*Ornithodoros sp larval form*	18
	Spinturnicidae	*Ancystropus sp1*	8

Table 3.2: contd…

***Miniopterus schreibersii* – Wavulgalge**

Host identity	Details of the Parasites		
	Family	**Species**	**# of Parasites**
WC/Ms/2	Nycteribiidae	*Penuicillida indica*	2
	Streblidae	*Brachytarsina ambionensis*	2
WC/Ms/3	Nycteribiidae	*Penuicillida indica*	1
	Nycteribiidae	*Nycteribia allotopa*	1
	Streblidae	*Brachytarsina ambionensis*	1
WC/Ms/4	Streblidae	*Brachytarsina ambionensis*	1
WC/Ms/5	Nycteribiidae	*Nycteribia allotopa*	1
	Streblidae	*Brachytarsina ambionensis*	1
	Spinturnicidae	*Spinturnix psi*	1
WC/Ms/6	Nycteribiidae	*Nycteribia allotopa*	1
	Streblidae	*Brachytarsina ambionensis*	1
	Spinturnicidae	*Spinturnix psi*	1
WC/Ms/7	Nycteribiidae	*Nycteribia sp1*	2
	Nycteribiidae	*Penuicillida indica*	5
	Streblidae	*Brachytarsina ambionensis*	1
WC/Ms/8	Streblidae	*Brachytarsina ambionensis*	7
WC/Ms/9	Streblidae	*Brachytarsina ambionensis*	1
WC/Ms/10	Nycteribiidae	*Nycteribia sp1*	6
	Nycteribiidae	*Penuicillida indica*	2
	Spinturnicidae	*Spinturnix psi*	3

***Hipposideros speoris* – Wavulgalge**

Host Identity	Details of the Parasites		
	Family	**Species**	**# of parasites**
WC/Hs/5	Intestinal treamatoda	Unidentified	1

***Megaderma spasma* – Galapatha**

Host Identity	Details of the parasite		
	Family	**Species**	**# of parasites**
5	Intestinal treamatoda	Unidentified	1

***Hipposideros speoris*- Kurunegala House**

Host Identity	Details of the parasite		
	Family		**Family**
KGH/Hs/1	Flies	Unidentified	1

***Hispposideros ater* - Kurunegala House**

Host Identity	Details of the parasite		
	Family	**Species**	**Family**
KGH/Ha/1	Intestinal treamatoda	Unidentified	1

Nycteribiids were recorded from 33 individuals of bats whereas Streblids were recorded from 17 individuals. There were four species of Nycteribids, (*Eucampsipoda latisterna, Stylidia phillipsi, Nycteribia allotopa* and *Penuicillida indica). E. latisterna* was the most abundant bat fly recorded for this family. 73% of Nycteribids were from this species followed by *S. phillipsi* (13%), *P. Indica* (11%) and *N. allotopa* (3%).

Nycteribids were recorded from 3 species of bats, *R. leschenaulti* (both from Naugala cave and Wavulgalge), *R. rouxii* (from Wavulgalge and Muththtettuwegama house) and *M. schreibersii* from Wavulgalge (Fig. 3.2). They were recorded from all species of bats recorded from Wavul Galge, which was a natural cave. In addition bat flies were recorded from Naugala cave and Muththettuwegama house. Very high presence of parasitic flies were recorded from *R. leschenaulti* captured from Naugala cave.

Mites (Spinturinidae) were recorded from 20 individuals, the host species being *R. leschenaulti, M. schreibersii* and *R. rouxii.* They were recorded from those bats species, which were captured from caves. Highest number (85%) of mites was recorded from *R. leschenaulti* (52% from bats screened from Naugala cave and 33% from those recorded from Wavulgalge.) (Figure 3.3).

Four species of mites were recorded in this study; *Ancystropus* sp, *Paraperoghichus* sp. *Onchocelus kanhei* and *Spinturix psi.* 76% of mites were *Ancystropus*, recorded only in *R.leschenaulti.*

Streblid flies were recorded from 3 species; *R. rouxii* from Muththettuwegama house, *R. leschenaulti* from both Naugala cave and Wavulgalge, and *M. schreibersii* in Wavulgalge (Fig. 3.4). The parasites showed high host specificity as 4 different species of parasites were recorded from 4 species of host bats: *Brachytarsina ambionensis* from *M. schreibersii, Ramondia pagodarum* from *R. leaschenaulti* from Wavulgalge, *Megasterbla* species from *R. leschenaulti* in Naugala cave and unidentified *Brachytarsina sp.* from *R. rouxii* in Muththettuwegama house.

A larval form of *Ornithodoros sp* of the family Argasidae was recorded from *R. leschenaulti* in both Naugala and Wavulgalge (Fig. 3.5). Very high parasite infestation was found in these bats as 54 parasites were recorded from 8 individuals and in one host species from Naugala cave, *18* parasites were screened. (Table 3..2).

Haemaphysalis larval form of the family Ixoididae was recorded from *R. rouxii* in Wavulgalge. Very high parasitic infestation was observed for *R. leschenaulti* from Naugala cave. Parasites were recorded from all specimens screened.

3.3. Host specificity

High host specificity was seen in almost all parasites recorded. *E. latistrana* was recorded only from *R. leschanaulti* captured in both Wavulgalge and Naugala cave (Table 3.2). Although, Wavulgalge was a sympatric roost and another 3 species of bats (*R. rouxii, M. schreibersii* and *H. speoris*) were screened for parasites in this study, not a single individual of *E. latistrana* was recorded in any of these bat species or even in any other species examined for this study. *S. phillipsi* was recorded only in *R. rouxii* and was recorded in bats in both Muththettuwegama and Wavulgalge (Table 3.2). *N. allotopa* and *P. indica* were recorded only in *M. schreibersii*.

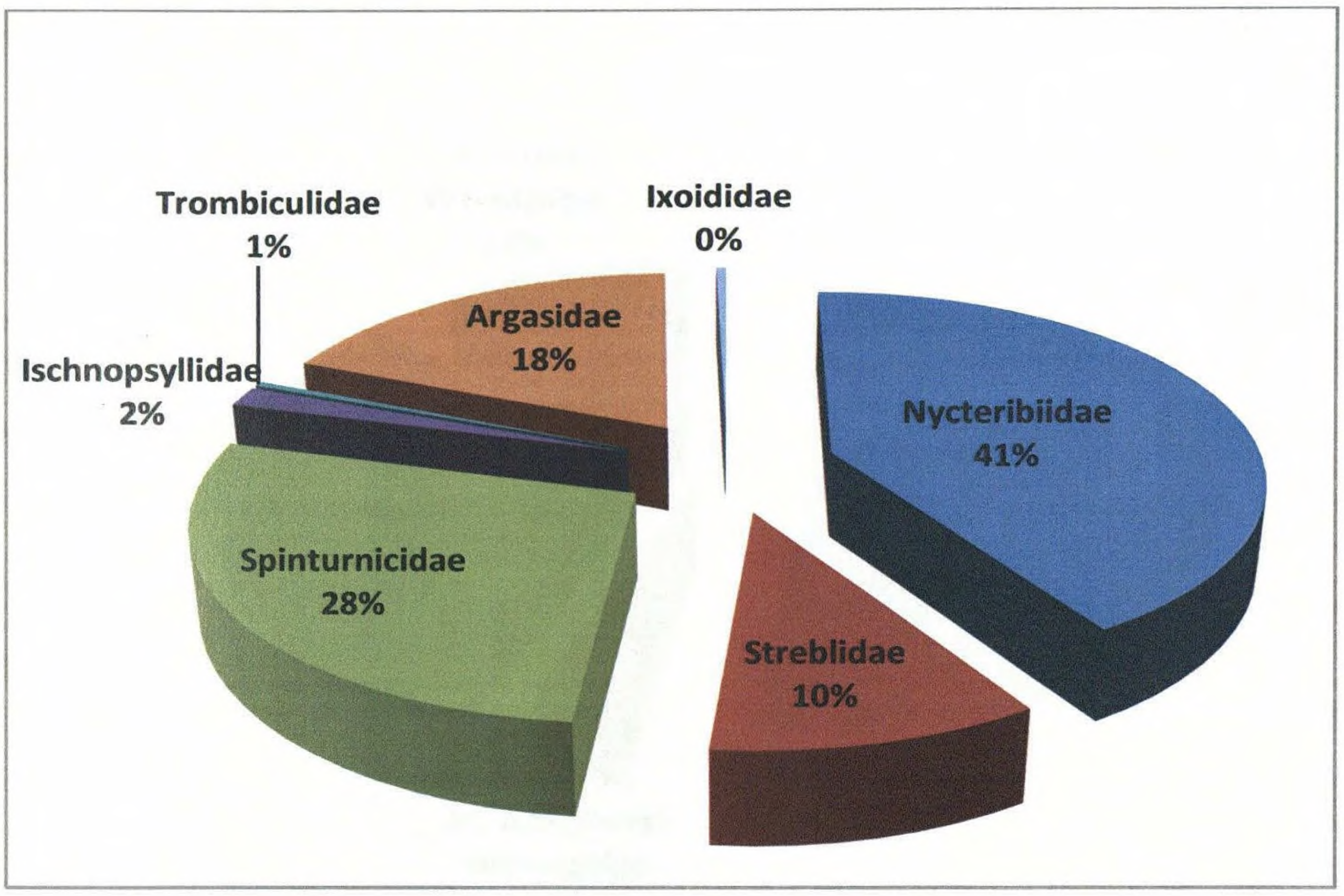


Figure 3.1. Percentage of ecto-parasites recorded from different families during the study. About 70% of the parasites were recorded from two families, Nycteribiidae and Spinturnicidae.

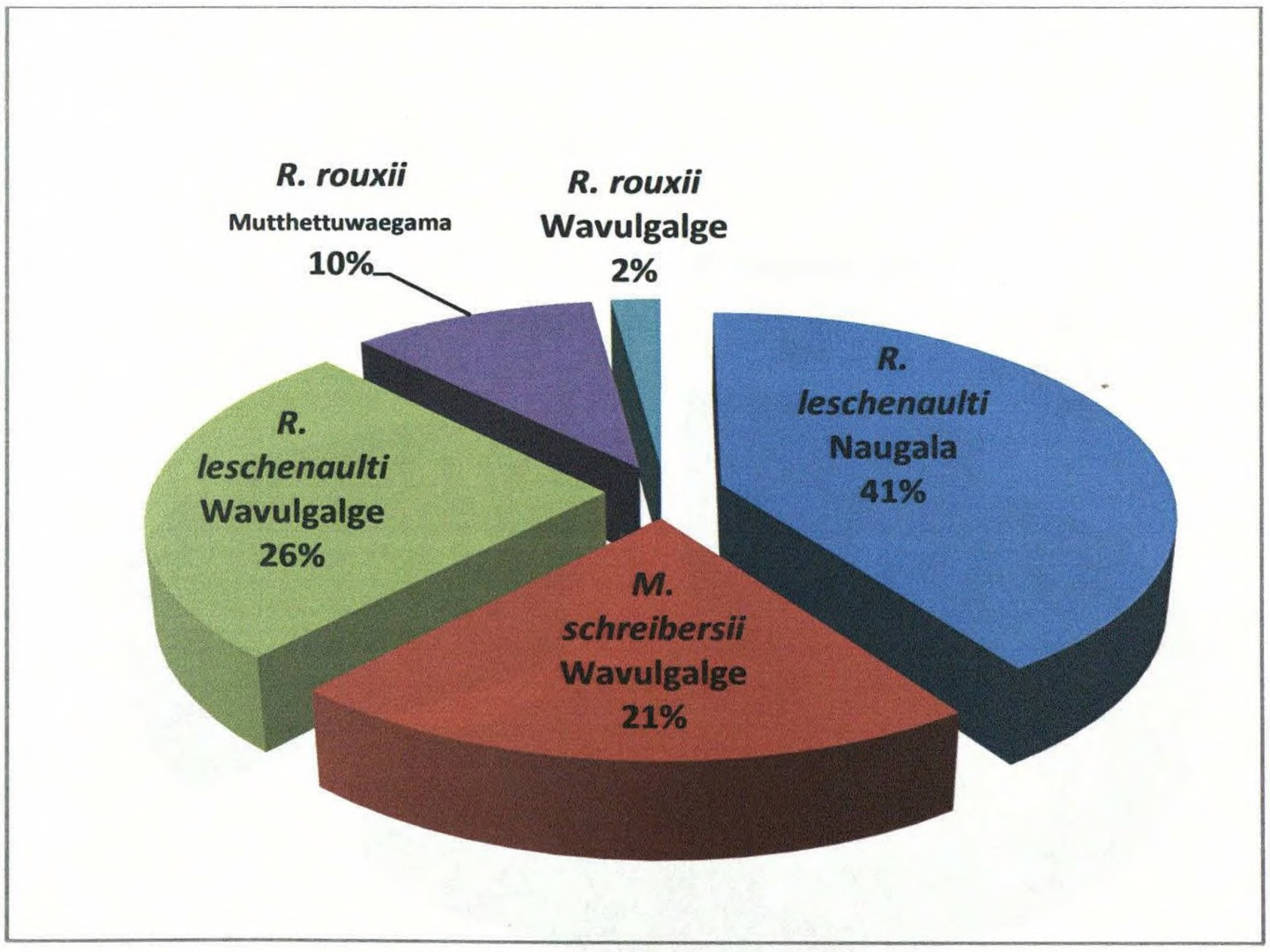


Figure 3.2. Percentage of Nycteribid parasites recorded from different host species at different roosting sites.

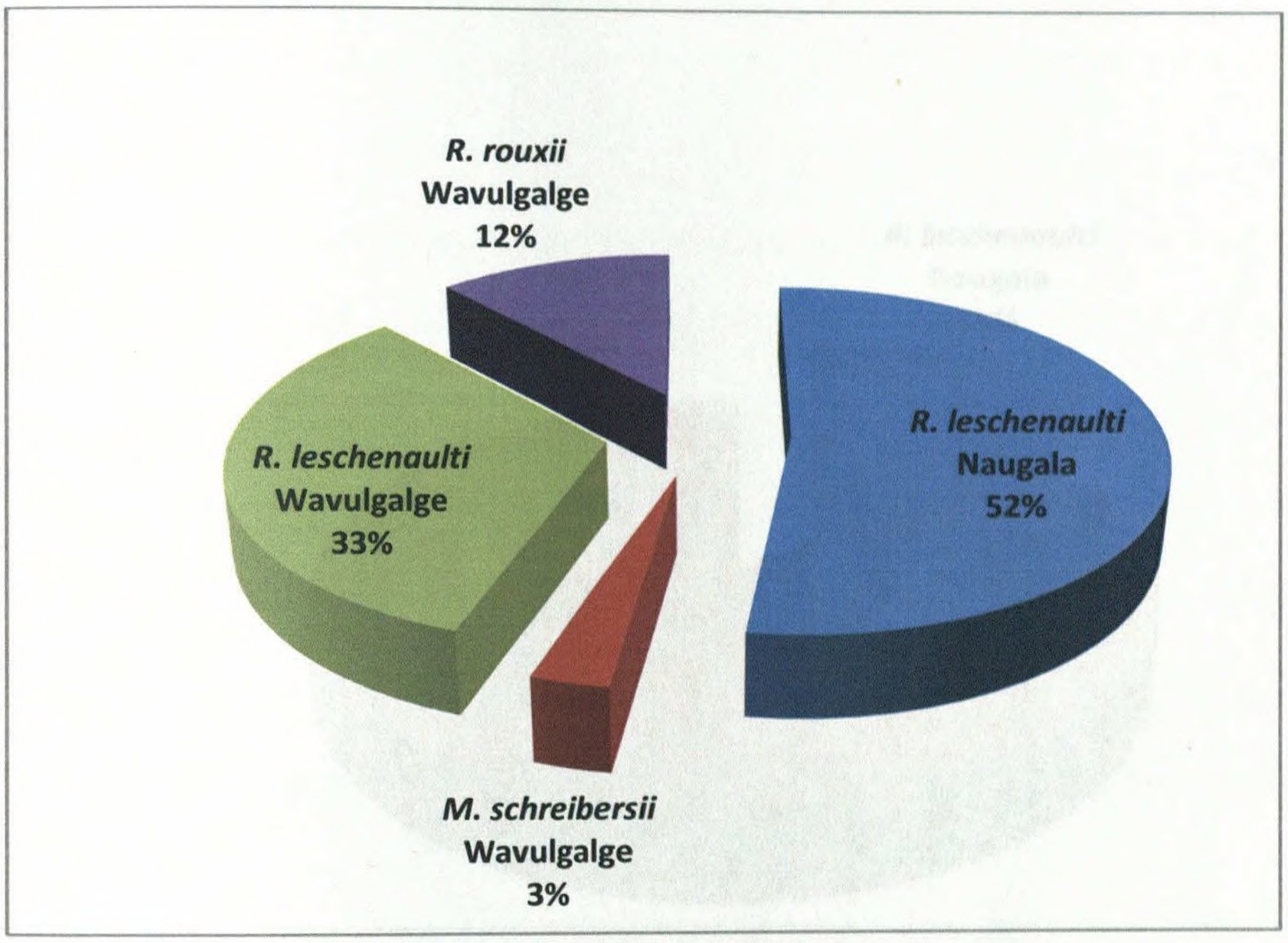


Figure 3.3. Percentage of Spinturnicid parasites recorded from bat species captured from different roosting sites. Over 50% of mites were recorded from *R. leschenaulti* captured from Naugala cave. Note 85% of mites were recorded from the fruit bat, *R. leschenaulti*.

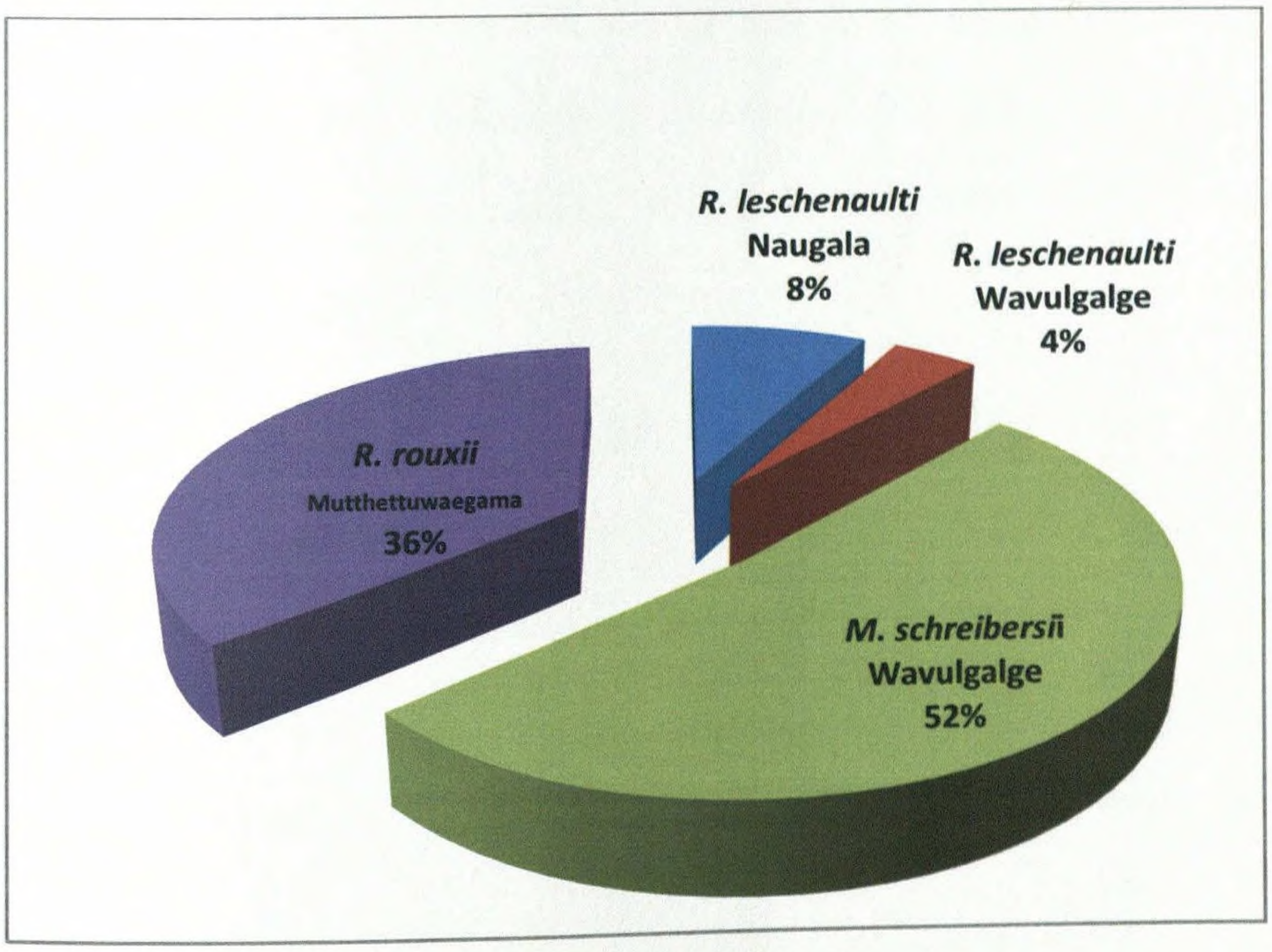


Figure 3.4. Percentages of parasites of Family Steblidae by host species and roosting sites.

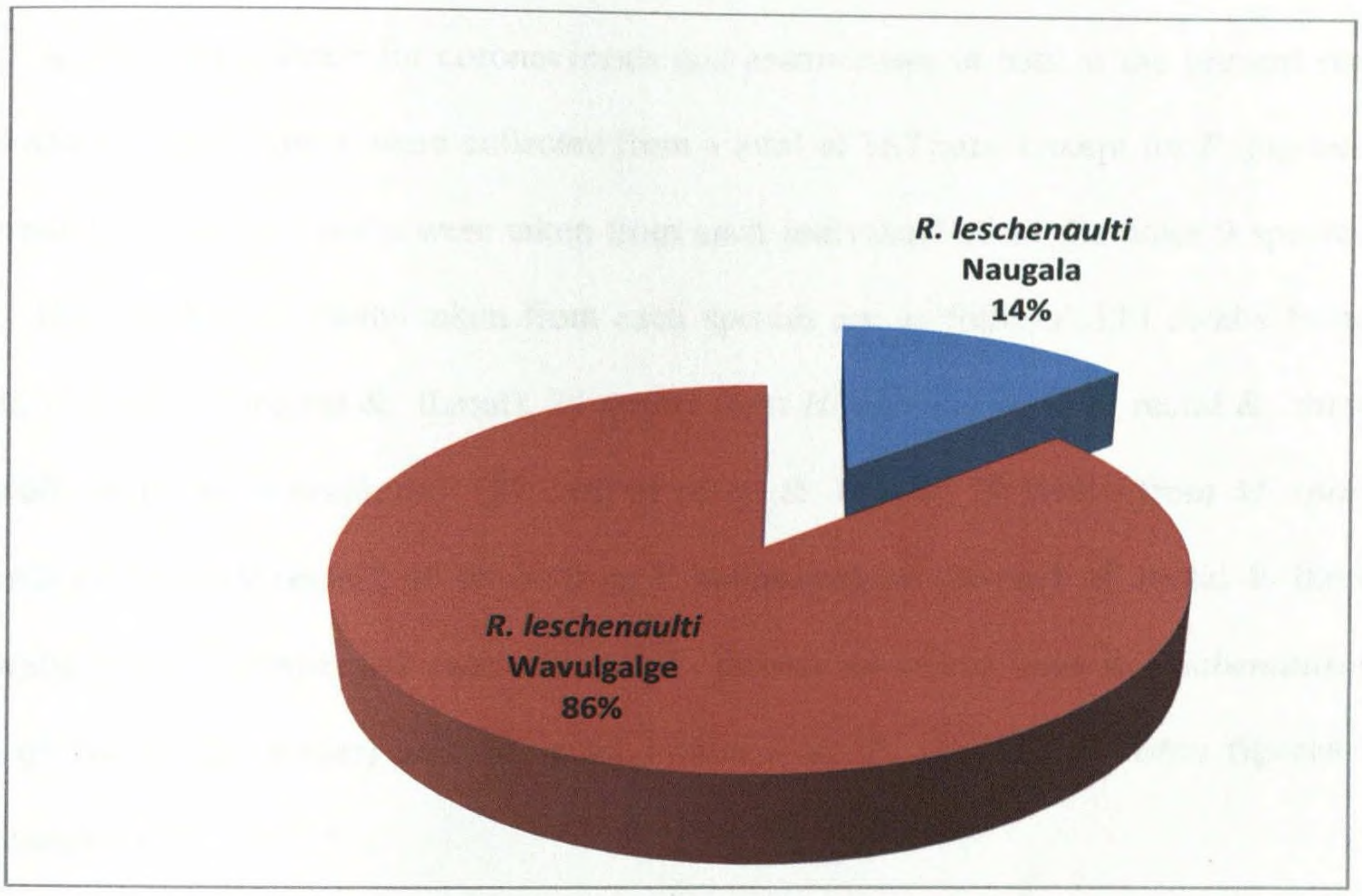


Figure 3.5. A larval form of *Ornithodoros* sp. (Family Argasidae) was recorded only from the fruit bat *R. leschenaulti*. Over 86% of parasites were recorded from Wavulgalge.

3.4. **Throat and Rectal Samples**

In the surveillance for coronaviruses and astroviruses in bats in the present study, rectal and oral specimens were collected from a total of 367 bats. Except for *P. giganteus*, both rectal and throat swabs were taken from each individual of all the other 9 species of bats. The number of swabs taken from each species are as follows: 114 swabs from *R. rouxii*, (57 each of rectal & throat), 24 swabs from *H. ater* (12 each of rectal & throat), 54 swabs from *M. schreibersii* (27 each of rectal & throat), 20 swabs from *M. spasma* (10 each of throat & rectal), 40 swabs from *T. melanopogon* (20 each of rectal & throat), 64 swabs from *C. sphinx* (32 each of rectal & throat), 44 swabs from *R. leschenaulti* (22 each of rectal & throat) and 40 rectal swabs from *P. giganteus*. These figures are summarized in Table 3.3.

With the pan-coronavirus and pan-astrovirus RT-PCR assays, two coronavirus positives and 14 astrovirus positives were detected from the samples (Table 3.4). Coronaviruses were found only from *M.* schreibersi captured from Wavulgalge. The astroviruses were found from *R.rouxii, H. ater, H. speoris, M. spasma, M. schreibersii, H. lankadiva* and *R. leschenaulti.* Viruses were not recorded at all from three species of bats: *C. sphinx (captured from two locations), P. giganteus (3 locations)* and *Taphozous melanopogon* (single location).

Astrovirus were found from 23 individuals (Table 3.4). Highest number of positives / roost was recorded from Galapatha temple (for *M. spasma)* and Wavulgalge (*M. schreibersii).* In both of these locations 50% from those screened were positive for astrovirus (Table 3.4). *R. leschenaulti* was sampled from three locations and although astrovirus was recorded from two of these locations (Naugala cave and Wavulgalge), bats screened from the colony at National Museum did not carry any viruses. Horseshoe bats *(R. rouxii)* captured from both Wavulgalge and Muththetuwegama House were positive for astroviruses. Similarly virus positive individuals were recorded from 5 locations, which included 3 buildings (Muththettuwegama house, Kurunagala house, Galapatha temple) and two caves (Wavulgalge and Naugala cave). Out of these, Muthettuwegama house and Galapatha temple were single species colonies. In sympatric roosts (Wavulgalge and Kurunagala House) virus positive individuals were recorded from all sympatric species. In Wavulgalge, the three species screened *(R. leschenaulti, M. scheribersii and R. rouxii)* were positive for astrovirus and also cornoavirus and both *H. ater* and *H. speoris* recorded from Kurunagala house were also positive for astrovirus.

These PCR amplicons were sequenced for further analysis. Phylogenetic studies have shown that all these astroviruses and coronaviruses detected were novel but related to viruses detected from bats from other countries in Asia, Australia and Europe (Figures 3.6 and 3.7).

Both coronaviruses recorded from Sri Lankan samples were from *M. schreibersii,* captured from the same day roosts (Wavulgalge). Phylogenetic analysis of P gene showed that both of these viruses are novel and differ from one another and cluster into two different groups (Fig. 3.6).

Table: 3.3 . The details of the swabs taken from different species of bats at different roosting sites. (S: Saliva; F: Fecal).

	Site Visit No.	*R. rouxii*		*H. ater*		*H.lankadiva*		*H.speoris*		*M.schreibersii*		*M. spasma*		*T. melanopogon*		*C.sphinx*		*R.leschenaulti*		*P. giganteus*	
		S	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**
Wavul Galge	1	10	10					6	6	10	10							10	10		
	2	5	5					7	7	6	6							2	2		
	3	10	10							11	11										
Naugala Cave	1																	10	10		
Fashiengala Cave	1							7	7					8	8						
	2							7	7					6	6						
	3							7	7					6	6						
Gaalapatha Temple	1											10	10								
Kurunagala house	1	10	10	6	6			8	8												
	2	2	2	6	6			8	8												
	3							8	8												
Muththettu-wegama house	1	10	10																		
	2	10	10																		
Bogala Mine	1					10	10														
	2					9	9														
Uni Of Colombo	1															12	12				
Jaela	1															10	10				1
Hunupitiya	1																				1
Vihara Maha Devi Park	1																				1
	2																				1
National Museum	1															10	10				

Table 3.4. Details of the astroviruses and corona viruses recorded from different species of bats

Species	**No. of bats Screened**	**AstV**	**CoV**
Cynopterus sphinx	44	0	
Hipposideros ater	23	1	
Hipposideros lankadiva	29	1	
Hipposideros speoris	77	2	
Megaderma spasma	18	1	
Miniopterus schreiberisii	38	5	2
Pteropus giganteus	30	0	
Rhinolophus rouxii	61	3	
Rousettus leschenaulti	31	1	
Taphozous melanopogon	16	0	
Total:	**367**	**14**	**2**

Except the astrovirus recorded from *R. leschenaulti*, all other astroviruses recorded from bats of the same genus from different regions world-wide, clustered together to form monophyletic groupsn (Figure 3.7). For example, the astroviruses recorded from *M. schreibersii* from Sri Lanka are clustered with other species of *Miniopterus* examined from Hong Kong and China.

Among microchiropterans, astroviruses from *Miniopterus schreibersii* and *Megaderma spasma,* were clustered together. Viruses recorded from *R. rouxii, H. speoris* and *H. atrer* are also closely related and separated from *Miniopterus* group (Fig 3.7).

The astroviruses recorded from Sri Lankan bats separate into two main groups. All microchiropterans into one and those recorded from the fruit eating bat, *R. leschenaulti* into the other (Fig. 3.7), Infact the astrovirus recorded from *R. leschenaulti* from Wavulgalge (SL99 F- Figure 3.7) differed from the astroviruses recorded from all other microchiropteran bats. Infact this astrovirus is clustered among the astrovirus recorded from other major groups of mammals such as dolphins, sealions and cheetah (Fig. 3.8).

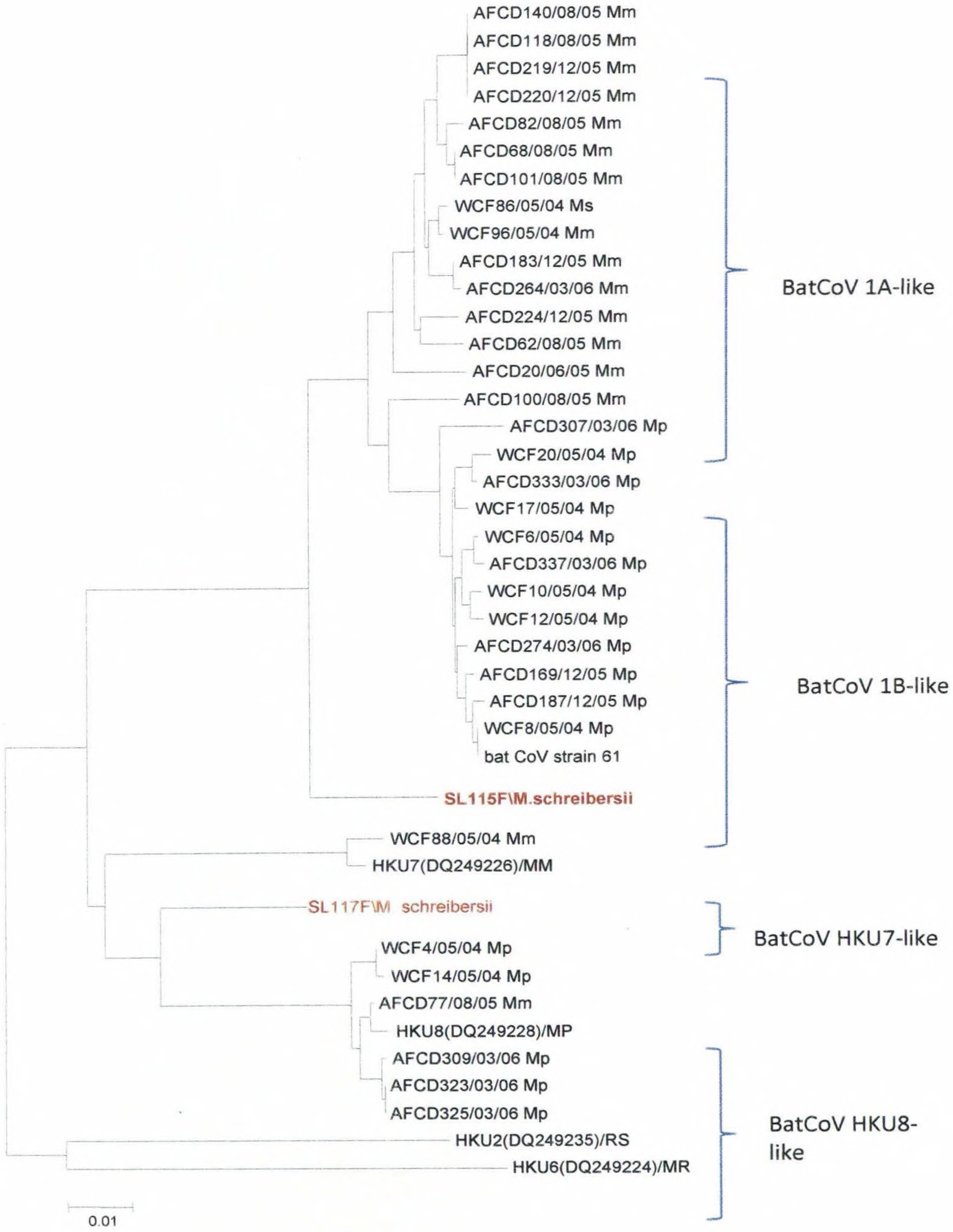


Fig. 3.6. Phylogenetic analysis on P gene of coronavirus from bats (Sri Lanka and Hong Kong). Viruses from Sri Lanka samples are in bold red font.

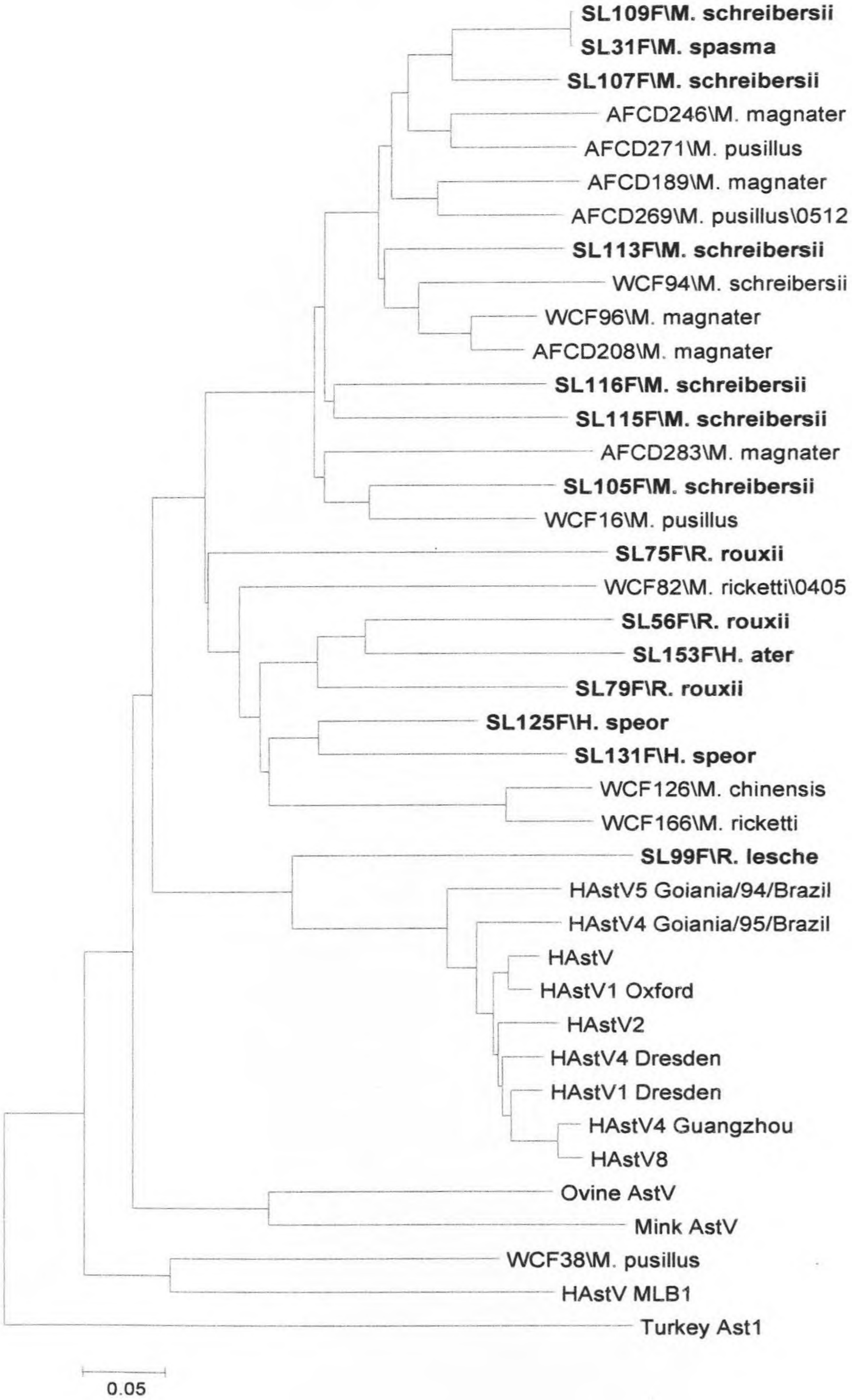
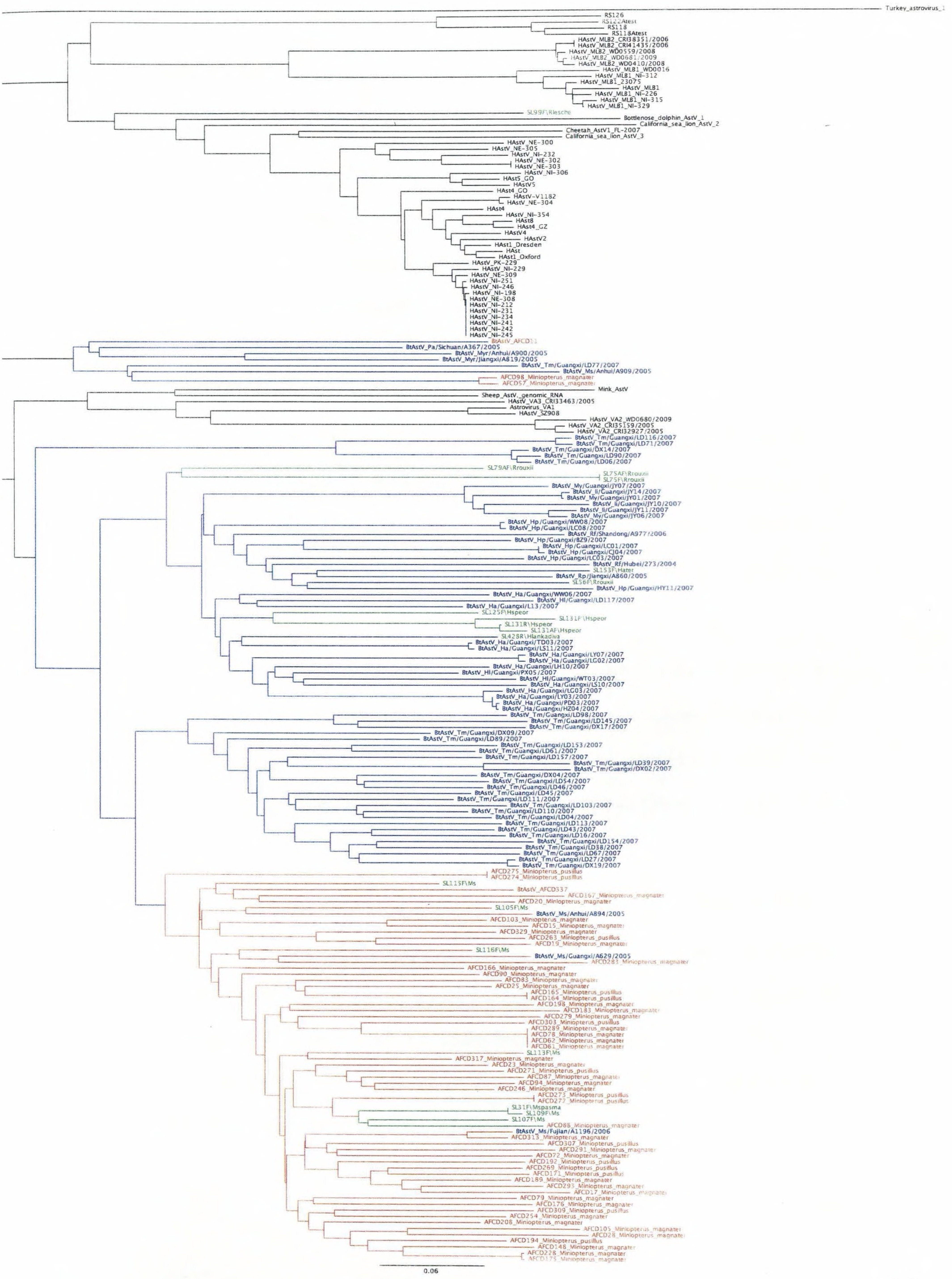


Figure 3.7. Phylogenetic analysis on P gene of astroviruses from bats (Sri Lanka and Hong Kong) and other animals. Viruses from Sri Lankan samples are in bold font.

Figure 3.8. Phylogenetic relationship of astroviruses recorded from different species of animals. Note that the astrovirus recorded from the Sri Lankan fruit bat SL 99/Green Font (*R. leschenaulti)* clusters with other groups of mammals and not with any of the other microchiropteran bat species.


Turkey_astrovirus_1
RS126
RS122Atest
RS118
RS118Atest
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HAstV_MLB2_CRI41435/2006
HAstV_MLB2_WD0559/2008
HAstV_MLB2_WD0681/2009
HAstV_MLB2_WD0410/2008
HAstV_MLB1_WD0016
HAstV_MLB1_NI-312
HAstV_MLB1_23075
HAstV_MLB1
HAstV_MLB1_NI-226
HAstV_MLB1_NI-315
HAstV_MLB1_NI-329
SL99F\Rlesche
Bottlenose_dolphin_AstV_1
California_sea_lion_AstV_2
Cheetah_AstV1_FL-2007
California_sea_lion_AstV_3
HAstV_NE-300
HAstV_NE-305
HAstV_NI-232
HAstV_NE-302
HAstV_NE-303
HAstV_NI-306
HAst5_GO
HAstV5
HAst4_GO
HAstV-V1182
HAstV_NE-304
HAst4
HAstV_NI-354
HAst8
HAst4_GZ
HAstV4
HAstV2
HAst1_Dresden
HAst
HAst1_Oxford
HAstV_PK-229
HAstV_NI-229
HAstV_NE-309
HAstV_NI-251
HAstV_NI-246
HAstV_NI-198
HAstV_NE-308
HAstV_NI-212
HAstV_NI-231
HAstV_NI-234
HAstV_NI-241
HAstV_NI-242
HAstV_NI-245
BtAstV_AFCD11
BtAstV_Pa/Sichuan/A367/2005
BtAstV_Myr/Anhui/A900/2005
BtAstV_Myr/Jiangxi/A819/2005
BtAstV_Tm/Guangxi/LD77/2007
BtAstV_Ms/Anhui/A909/2005
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AFCD57_Miniopterus_magnater
Mink_AstV
Sheep_AstV,_genomic_RNA
HAstV_VA3_CRI33463/2005
Astrovirus_VA1
HAstV_SZ908
HAstV_VA2_WD0680/2009
HAstV_VA2_CRI35159/2005
HAstV_VA2_CRI32927/2005
BtAstV_Tm/Guangxi/LD116/2007
BtAstV_Tm/Guangxi/LD71/2007
BtAstV_Tm/Guangxi/DX14/2007
BtAstV_Tm/Guangxi/LD90/2007
BtAstV_Tm/Guangxi/LD06/2007
SL79AF\Rrouxii
SL75AF\Rrouxii
SL75F\Rrouxii
BtAstV_My/Guangxi/JY07/2007
BtAstV_Ii/Guangxi/JY14/2007
BtAstV_My/Guangxi/JY01/2007
BtAstV_Ii/Guangxi/JY10/2007
BtAstV_Ii/Guangxi/JY11/2007
BtAstV_My/Guangxi/JY06/2007
BtAstV_Hp/Guangxi/WW08/2007
BtAstV_Hp/Guangxi/LC08/2007
BtAstV_Rf/Shandong/A977/2006
BtAstV_Hp/Guangxi/BZ9/2007
BtAstV_Hp/Guangxi/LC01/2007
BtAstV_Hp/Guangxi/CJ04/2007
BtAstV_Hp/Guangxi/LC03/2007
BtAstV_Rf/Hubei/273/2004
SL153F\Hater
BtAstV_Rp/Jiangxi/A860/2005
SL56F\Rrouxii
BtAstV_Hp/Guangxi/HY11/2007
BtAstV_Ha/Guangxi/WW06/2007
BtAstV_Hl/Guangxi/LD117/2007
BtAstV_Ha/Guangxi/L13/2007
SL125F\Hspeor
SL131F\Hspeor
SL131R\Hspeor
SL131AF\Hspeor
SL428R\Hlankadiva
BtAstV_Ha/Guangxi/TD03/2007
BtAstV_Ha/Guangxi/LS11/2007
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BtAstV_Ha/Guangxi/LH10/2007
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AFCD274_Miniopterus_pusillus
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AFCD167_Miniopterus_magnater
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SL105F\Ms
BtAstV_Ms/Anhui/A894/2005
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AFCD15_Miniopterus_magnater
AFCD329_Miniopterus_magnater
AFCD263_Miniopterus_pusillus
AFCD19_Miniopterus_magnater
SL116F\Ms
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AFCD283_Miniopterus_magnater
AFCD166_Miniopterus_magnater
AFCD90_Miniopterus_magnater
AFCD83_Miniopterus_magnater
AFCD25_Miniopterus_magnater
AFCD165_Miniopterus_pusillus
AFCD164_Miniopterus_pusillus
AFCD198_Miniopterus_magnater
AFCD183_Miniopterus_magnater
AFCD279_Miniopterus_magnater
AFCD303_Miniopterus_pusillus
AFCD289_Miniopterus_magnater
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AFCD61_Miniopterus_magnater
SL113F\Ms
AFCD317_Miniopterus_magnater
AFCD23_Miniopterus_magnater
AFCD271_Miniopterus_pusillus
AFCD87_Miniopterus_magnater
AFCD94_Miniopterus_magnater
AFCD246_Miniopterus_magnater
AFCD273_Miniopterus_pusillus
AFCD272_Miniopterus_pusillus
SL31F\Mspasma
SL109F\Ms
SL107F\Ms
AFCD88_Miniopterus_magnater
BtAstV_Ms/Fujian/A1196/2006
AFCD313_Miniopterus_magnater
AFCD307_Miniopterus_pusillus
AFCD291_Miniopterus_magnater
AFCD72_Miniopterus_magnater
AFCD192_Miniopterus_pusillus
AFCD269_Miniopterus_pusillus
AFCD171_Miniopterus_pusillus
AFCD189_Miniopterus_magnater
AFCD293_Miniopterus_magnater
AFCD17_Miniopterus_magnater
AFCD79_Miniopterus_magnater
AFCD176_Miniopterus_magnater
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AFCD105_Miniopterus_magnater
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AFCD228_Miniopterus_magnater
AFCD175_Miniopterus_magnater
0.06

Figure 3.8

4. Discussion

Two coronaviruses and 14 astroviruses were detected from the samples analysed from Sri Lanka. This is the first record of the presence of viruses in a Sri Lankan bat population. Both coronaviruses were detected from *M. schreiberssii* captured from Wavulgalge. Phylogenetic analysis of coronaviruses from bats and other mammals has shown that these viruses in bats can be found in several major groups of mammalian species including humans (Lau et al. 2005). As shown in our study these viruses are most similar to HKU8 and bat CoV 1 which were also detected from *Miniopterus* bats in Hong Kong. This also shows that despite geographic differences, the viruses from host of the same genus clusters together. Similar observations have been made by Gouth *et. al.* (2011) who demonstrated the similarity between the corona viruses found in South East Asia and Africa. Our results also confirm that bats are also a natural reservoir for mammalian coronaviruses in nature.

In our study, we recorded 14 astroviruses, in addition to two coronaviruses. Detection of astroviruses have so far been reported only from insectivorous bats in Hong Kong and Mainland China (Chu et al. 2008). In the present study, we have recorded a new astrovirus from a bat species in Sri Lanka, namely *R. leschenaulti* captured from Wavulgalge.

Detection of this virus from a fruit bat is of significance, since most fruit eating bats chew the fruits to swallow sugar and other nutrients and then spit out partially digested fruits. Other animals may ingest these fruit remnants and may consequently become infected with virus particles in residual bat saliva (Dobsan, 2005). In many areas of the world, specially in Sri Lanka large areas of natural habitats are converted to agricultural lands. And surviving bat populations will concentrated in the remaining patches of forests or forced to live in the modified ecosytems. When these patches are used for shade or feeding grounds for domesticated animals such as cattle and buffalos food remnants (insect as well as fruits) chewed bats may find their way into human food chain (Dobson, 2005).

The astrovirus recorded from *R. leschenaultia* has shown a unique relationship to typical human astroviruses. Moreover, no astrovirus from a bat species relating to any other group of mammal has previously been recorded. Astrovirus recorded from the fruit eating bat, *R. leaschenaulti* from Wavulgalge (SL99F- Figure 3) is clustered among the astrovirus recorded from other major groups of mammals such as dolphins, sealions and cheetah (Fig. 3.8). This not only suggests that fruit bats also carry astroviruses, but also these can be more related to previously known astroviruses detected in other mammals including humans, porcine, rats. There has to be a source for astroviruses in these groups of mammals, including humans, and this finding of an astrovirus on top of the phylogenetic tree, makes us wonder whether *R. leschenaulti* (and perhaps other fruit eating bats) are the reserviour for astroviruses in mammals. Undoubtedly more intense sampling is needed to elucidate this fact. Further surveillance of fruit eating bats, specially *R. leaschenaulti*, will provide a more complete picture on the ecology of astroviruses in all other mammals.

Apart from the astrovirus recorded from *R. leschenaulti*, (as shown in the Figure 3), phylogenetic analysis of astroviruses from bats of the same genus from different regions was seen to cluster together, to form monophyletic groups. For example, the astroviruses recorded from *M. schreibersii* from Sri Lanka are clustered with *M. pusillus* and *M. magnate* from Hong Kong suggesting these virus groups are host-genus specific.

Three batches of samples were sent to University of Hong Kong for analysis. In the first batch of samples from 77 bats, we have recorded 2 coronaviruses and 13 astroviruses. Thus, we have obtained 15% astrovirus positive samples and 1% coronavirus positives. However, most of the PCR amplicons were relatively weak. Therefore, we changed the collection media for the second batch of samples (RNA later), but when the analysis was done there had been no positive results at all. Thus for the 3rd batch (n=90) we reverted back to the original media and we have recorded only a single astrovirus from *R. leschenaulti.* The failure to record positive results in the second media may be due to a problem with the collection media or the aluminium-calcium alginate swab used, which would have produced inhibitor for downstream RT-PCR. However, this was a major set back in our study as this batch (n = 200) was the largest of the 3 batches were have collected and analysed.

Although a large number of parasites were recorded from the bat species screened in this study, we did not find any significant association between the presence of parasites and presence of viruses. In Naugala cave, in which we have seen highest parasitic infestation, an astrovirus was recorded only from one individual of *R. leschenaulti* and a single Nycteribid fly (*E. latisterna)* was recorded from this bat. However, same parasite fly and several other parasites were recorded from all other host bat species sampled in this cave, though they were negative for any viruses. In Galapatha Temple, we did not record any parasites in 10 bats (*M. spasma*), yet we found 3 positives for astroviruses. Highest positive was recorded for *M. schreibersii* from Wavulagalge. Out of the 38 bats screened, 5 astroviruses and 2 coronaviruses were recorded from this species. A steribid fly, *B. ambinensis was recorded* from all individuals, which was positive for viruses. However, the same parasite was also recorded in other bats, which were negative for any of the viruses. Thus we did not find any noteworthy association between the ectoparasites and the presence or absence of astro- and -corona viruses.

· Bats were captured from 12 roosts, which included 4 buildings, 4 natural caves and 4 open roosts. Viruses were recorded from both caves and buildings, although no viruses were recorded from those captured from open roosts. It should also be noted that viruses were not recorded from some of the caves (Fashiengala cave) or some buildings (National Museum). Thus, we do not see any significant association between the roost types and the detection of viruses. However, higher percentage of virus positive species were recorded from Wavulgalge, which is a larger sympatric cave. We have recorded four species of bats from this cave and we have recorded positive results for 3 species (only exception being *H. speoris*). Further more, over 50% of astroviruses (9/19) and 100% corona viruses were detected from this cave. Moreover, a unique astrovirus that we have isolated from *R. leschenaulti* was also recorded form Wavulgalge. This cave is one of the largest natural caves in Sri Lanka (Yapa *et.al.* 2000) and provides shelter for 5 species of bats (4 .microchiroperans and 1 megachiroperan). Thus this natural bat roost is an ideal site for future investigations on bat viruses in Sri Lanka.

Our findings of novel coronaviruses and astroviruses in bats in Sri Lanka provide information on the ecology of these viruses in bats in this country. When integrated with similar studies of bats in other continents, the results provide a wider picture on the ecology of the viruses in nature.

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Identification of a Novel Coronavirus in Bats

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Exotic wildlife can act as reservoirs of diseases that are endemic in the area or can be the source of new emerging diseases through interspecies transmission. The recent emergence of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) highlights the importance of virus surveillance in wild animals. Here, we report the identification of a novel bat coronavirus through surveillance of coronaviruses in wildlife. Analyses of the RNA sequence from the ORF1b and S-gene regions indicated that the virus is a group 1 coronavirus. The virus was detected in fecal and respiratory samples from three bat species (*Miniopterus* spp.). In particular, 63% (12 of 19) of fecal samples from *Miniopterus pusillus* were positive for the virus. These findings suggest that this virus might be commonly circulating in *M. pusillus* in Hong Kong.

Coronaviruses are positive-stranded RNA viruses. The viral genomes are between 29 and 32 kb long and are packaged in enveloped virions with corona-like morphology (5). The viral genomes contain five major open reading frames (ORFs) that encode the replicase polyproteins (ORF1a and ORF1ab), the spike (S), envelope (E), and membrane (M) glycoproteins, and the nucleocapsid protein (N) (5, 10). Of these proteins, the replicase polyproteins are directly translated from the viral genome. Translation of ORF1a-encoding sequence can be extended with ORF1b-encoding sequences by a −1 ribosomal frameshift to synthesize ORF1ab polyprotein (5). By contrast, other viral proteins are translated from subgenomic mRNA molecules that are synthesized by a discontinuous RNA synthesis mechanism (14, 22). The ORF1a and ORF1ab polyproteins are nonstructural proteins (6) and are cleaved by papain-like cysteine and 3CL proteinases to generate functional units for viral transcription and replication (26). S, E, M, and N proteins are structural proteins. S is responsible for receptor binding (4). E and M are integral membrane proteins and are the minimal set of proteins for virus assembly (1). N protein is an internal protein, and it binds to viral RNA to form ribonucleoprotein complex (11).

The majority of coronaviruses are disease-causing agents (16). Human coronaviruses are associated with respiratory and gastrointestinal diseases, and animal coronaviruses cause severe respiratory, enteric, neurological, or hepatic disease in their hosts. On the basis of antigenic and genetic analyses, coronaviruses are subdivided into three groups (groups 1 to 3) (5, 7). Group 1 viruses include human coronaviruses NL63 (HCoV-NL63) and 229E (HCoV-229E), canine coronavirus (CCoV), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and feline infectious peritonitis virus (FIPV). Group 2 viruses include human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), and murine hepatitis virus (MHV). Group 3 viruses are avian viruses, such as avian infectious bronchitis virus (IBV) and turkey coronavirus (TCoV).

Of the coronaviruses identified hitherto, most were isolated from humans, pets, pigs, cattle, or poultry. This bias is presumably because viral investigations are often driven by disease outbreaks in the above populations. By contrast, investigations of wildlife are rare (20, 24), and relatively little is known about the prevalence of coronavirus in wild animal species (3). The identification of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) in civet cats and other wild animals in live animal markets suggests that this novel human pathogen emerged as a result of an interspecies transmission (8). More importantly, these findings highlight the potential human health risk posed by coronaviruses in wild animals. This has prompted us to launch a survey of the prevalence of coronavirus in wild animals in Hong Kong. In particular, we were interested in determining whether wild animals living in this geographical region carry the precursor of SARS-CoV or other unidentified coronaviruses. Here, we report the identification of a novel bat coronavirus (BAT-CoV).

MATERIALS AND METHODS

Sample collection. The animal surveillance program was performed between the summer of 2003 and the summer of 2004. The study was approved and supported by the Department of Agriculture, Fisheries and Conservation, Hong Kong, Special Administrative Region, People's Republic of China. Small mammalian, avian, and reptile species living in natural reservoirs or country parks in Hong Kong were studied. Animals were trapped, and respiratory and fecal swab samples were collected. Before samples were taken, all animals were examined by a veterinary surgeon and confirmed to be free of overt disease. All captured animals were released after samples were taken. Samples were kept in viral transport medium (Earle's balanced salt solution, 0.2% sodium bicarbonate, 0.5% bovine serum albumin, 200 μg of vancomycin per liter, 18 μg of amikacin per liter, 160 U of nystatin per liter) at 4°C. In addition, blood samples were

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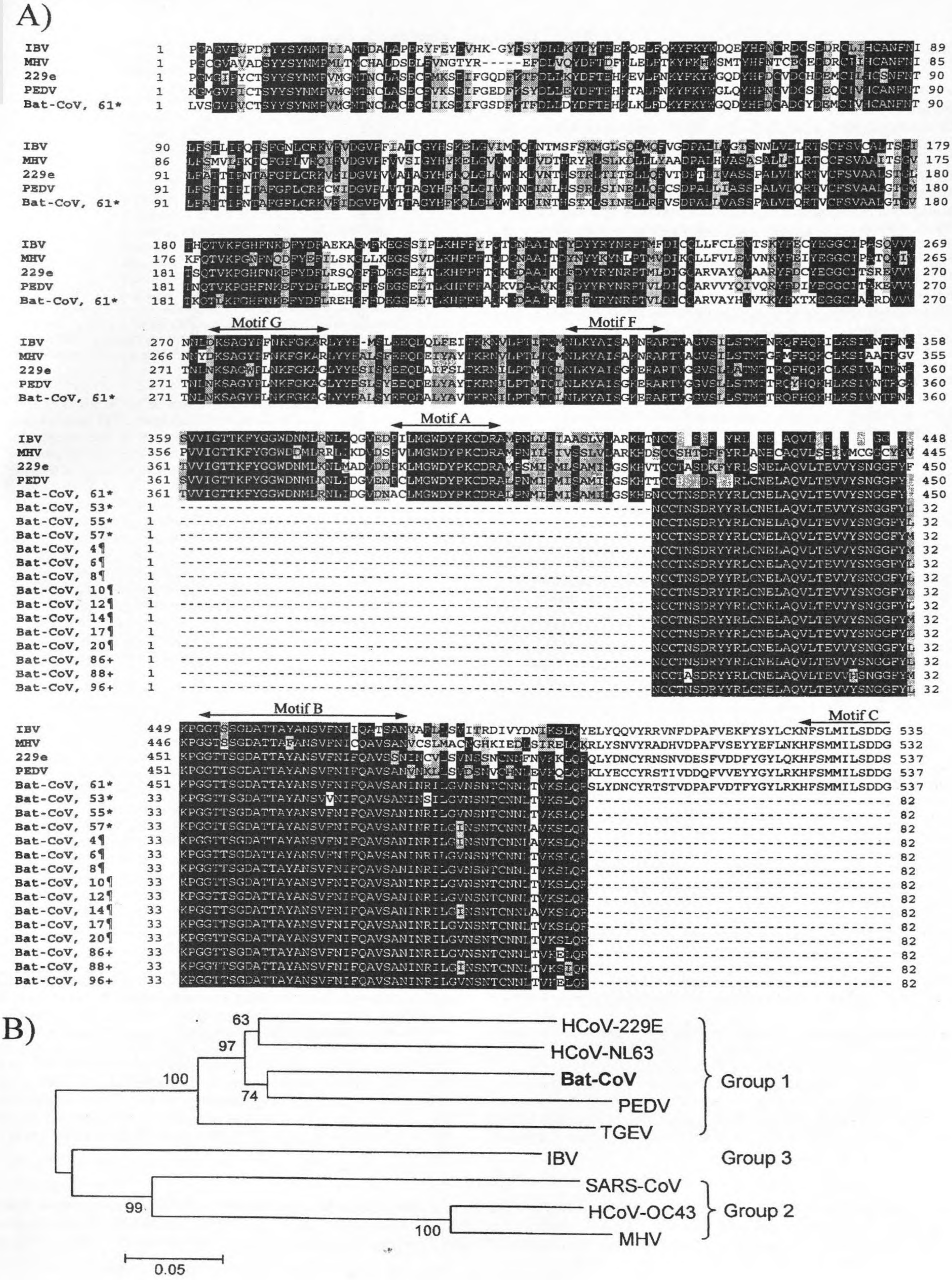

A)
IBV
MHV
229e
PEDV
Bat-CoV, 61*
Bat-CoV, 53*
Bat-CoV, 55*
Bat-CoV, 57*
Bat-CoV, 4¶
Bat-CoV, 6¶
Bat-CoV, 8¶
Bat-CoV, 10¶
Bat-CoV, 12¶
Bat-CoV, 14¶
Bat-CoV, 17¶
Bat-CoV, 20¶
Bat-CoV, 86+
Bat-CoV, 88+
Bat-CoV, 96+
Motif G
Motif F
Motif A
Motif B
Motif C
B)
63
97
100
74
99
100
HCoV-229E
HCoV-NL63
Bat-CoV
PEDV
TGEV
IBV
SARS-CoV
HCoV-OC43
MHV
Group 1
Group 3
Group 2
0.05

TABLE 1. Prevalence of Bat-Cov in animals

Animal type and scientific name	Common name	No. of individual animals	No. of positive respiratory samples	No. of positive fecal samples
Mammals				
Cynopterus sphinx	Short-nosed fruit bat	15	0	0
Hipposideros armiger	Great round-leaf bat	4	0	0
Hipposideros pomona	Bicolored round-leaf bat	3	0	0
Miniopterus magnater	Large bent-winged bat	16	1	2
Miniopterus pusillus	Lesser bent-winged bat	19	5	12
Miniopterus schreibersii	Japanese long-winged bat	4	1	1
Myotis myotis	Large mouse-eared bat	3	0	0
Myotis ricketti	Rickett's big-footed bat	5	0	0
Pipistrellus abramus	Japanese pipistrelle bat	3	0	0
Rhinolophus affinis	Intermediate horseshoe bat	2	0	0
Rhinolophus pusillus	Least horseshoe bat	1	0	0
Rhinolophus rouxi	Rufous horseshoe bat	6	0	0
Canis familiaris	Feral dog	6	0	0
Felis catus	Feral cat	1	0	0
Herpestes javanicus	Javan mongoose	1	0	0
Herpestes urva	Crab-eating mongoose	1	0	0
Hystrix hodgsoni	Chinese porcupine	10	0	0
Macaca mulatta	Rhesus macaque	6	0	0
Melogale moschata	Chinese ferret badger	8	0	0
Muntiacus muntjak	Indian muntjac	4	0	0
Paguma larvata	Himalayan palm civet	21	0	0
Rattus rattus	Black rat	6	0	0
Viverricula indica	Small indian civet	4	0	0
Sus scrofa	Wild boar	9	0	0
Reptiles				
Naja atra	Chinese cobra	1	0	0
Opisthotropis balteata	Banded stream snake	1	0	0
Ptyas korros	Indo-Chinese rat snake	2	0	0
Trachemys scrpta elegants	Red-eared slider	1	0	0
Rhabdophis subminiatus helleri	Red-necked keelback	1	0	0
Trimeresurus albolabris	Bamboo snake	1	0	0
Sibynophis chinensis	Chinese mountain snake	1	0	0
Sinonatrix percarinata	Mountain water snake	1	0	0
Elaphe radiata	Copperhead racer	1	0	0
Birds				
Chalcophaps indica	Emerald dove	1	0	0
Garrulax pectoralis	Greater necklaced laughing thrush	1	0	0
Garrulax perspicillatus	Masked laughing thrush	2	0	0
Myophonus caeruleus	Blue whistling thrush	3	0	0
Scolopax rusticola	Eurasian woodcock	1	0	0
Streptopelia chinensis	Spotted dove	3	0	0
Streptopelia orientalis	Oriental turtle dove	3	0	0
Turdus cardis	Japanese thrush	1	0	0
Turdus obscurus	Eyebrowed thrush	1	0	0
Turdus ohortulorum	Grey-backed thrush	1	0	0
Zoothera dauma	Scaly thrush	1	0	0

drawn from captured Himalayan palm civets for neutralization assays for SARS-CoV (8). The neutralization assays were performed as described previously (8).

RNA extraction and reverse transcription. RNA from 140 μl of the sample was extracted by QIAamp virus RNA mini kit (QIAGEN) following the manufacturer's instructions. Extracted RNA was eluted in 50 μl of RNase-free water and stored at −20°C. cDNA was generated as described previously (19). Briefly, 10 μl of eluted RNA samples was reverse transcribed by 200 U of Superscript II reverse transcriptase (Invitrogen) in a 20-μl reaction mixture containing 0.15 μg of random hexamers, 10 mmol of dithiothreitol per liter, and 0.5 mmol of deoxynucleoside triphosphate per liter. Reaction mixtures were incubated at 42°C for 50 min, followed by a heat inactivation step (72°C for 15 min). Reverse-transcribed products were stored at −20°C.

PCR and sequencing. A pair of consensus primers targeted to the conserved region of coronavirus RNA polymerase (RNA-dependent RNA polymerase [RdRp]) sequences was used to screen the RNA samples (PCR 2 in Table 2). In a typical PCR, 2 μl of cDNA was amplified in a 50-μl reaction mixture containing 0.2 mmol of deoxynucleoside triphosphates per liter, 3 mmol of $MgCl_2$ per liter, 0.5 μmol of forward primer per liter, 0.5 μmol of reverse primer per liter, and 0.25 U of AmpliTaq Gold (Applied Biosystems). PCR was performed as follows:

FIG. 1. (A) Protein sequence alignment of coronavirus RdRps. The conserved motifs for RdRps are indicated above the sequences. Specimens collected from *M. pusillus* at geographical site 1 (*), *M. pusillus* at geographical site 2 (¶), and *M. schreibersii* (specimen 86) and *M. magnater* (specimens 88 and 96) at site 3 (+) are indicated. 229e, HCoV-229E. (B) Phylogenetic analysis of RNA sequences encoding RdRp (partial sequence).

TABLE 2. Primer sequences positive for Bat-CoV detection

PCR	Target sequence	Primer orientation	Primer sequence (5′ to 3′)
1	RdRp	Forward	AYAACCAAGATCTTAATGG
		Reverse	TGCTTAGAACCCAAAATCAT
2[a]	RdRp	Forward	GGTTGGGACTATCCTAAGTGTGA
		Reverse	CCATCATCAGATAGAATCATCATA
3	Helicase-ExoN	Forward	CTCARGGTAGTGARTATGA
		Reverse	AATTGTTCWCCWGGTGG
4	Spike	Forward	WTATGTTTGYAATGGTAAY
		Reverse	GTCWTCATCMACWGTRC
5	Spike	Forward	GAYTDDCAGCACTTAATGC
		Reverse	TTGAGCCAYTCAAGRTYRA
6	Spike	Forward	CAATCTAGGTCTGCTATCG
		Reverse	CTAGAAGACTGTGATTTGA

[a] Forward and reverse primers (IN-6 and IN-7) were communicated through the World Health Organization's SARS etiology network by colleagues from the Centers for Disease Control and Prevention.

(i) 10 min at 95°C; (ii) 40 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. Amplified DNA products were analyzed by agarose gel electrophoresis and DNA sequencing. To avoid possible contamination, RNA extraction, reverse transcription-PCR (RT-PCR), and gel electrophoresis were preformed in separate laboratories. In addition, water controls were included in each run of the RT-PCR assay, and no false-positive result was observed in the negative-control reactions.

In subsequent experiments, primers targeted to the RdRp, helicase-ExoN, and S-encoding sequences were used to determine the Bat-CoV sequence (Table 2, PCRs 1 and 3 to 6). PCR conditions of these assays were identical to the PCR assay described above. DNA products with expected sizes were purified by QIAquick PCR purification kit (QIAGEN) and were cloned into DNA vectors (pCR-TOPO; Invitrogen). DNA inserts in purified plasmid were sequenced by BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were analyzed by ABI PRISM 3700 DNA analyzer (Applied Biosystems). Both sense and antisense sequences of these PCR products were sequenced at least once.

Data analysis. Deduced viral sequences were analyzed and aligned by BioEdit, version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees were constructed by the neighbor-joining method, and bootstrap values were determined by 1,000 replicates in MEGA 2.1 (http://www.megasoftware.net). Potential glycosylation sites of the S protein were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Stablecoil 1.0 (http://www.pence.ca/stablecoil/) was used to detect the heptad repeat (HR) regions of the S protein. Reference sequences used in the study are FIPV (GenBank accession number AB088222), TGEV (NC002306), HCoV-229E (AF304460), HCoV-NL63 (AY567487), HCoV-OC43 (NC005147), CCoV (AY342160), PEDV (AF353511), IBV (NC005147), MHV (NC001846), and SARS-CoV (NC004718).

Nucleotide sequence accession numbers. The deduced sequences from this study were deposited in GenBank under accession numbers AY864196 to AY864198.

RESULTS

Surveillance. A total of 162 swab samples from 12 bat species and 176 swab samples from 32 other animal species (Table 1) were collected. To identify coronaviruses from these specimens, a pair of consensus primers that can cross-react with a number of coronavirus RdRp sequences were used to screen the field samples (Table 2, PCR 2). Positive PCR amplicons were detected in three different bat species (*Miniopterus* spp. [Table 1]). In particular, 12 of 19 (63%) fecal swab samples from *Miniopterus pusillus* were positive in the screening test. These specimens were collected from three different geographical locations (Fig. 1A).

Identification of a Bat-CoV. To characterize the RdRp sequence deduced from PCR 2 (Table 2), amplicons from each animal were subjected to DNA sequencing. All the sequences generated from *M. pusillus* were highly similar (Fig. 1A). Although there are sequence polymorphisms among these sequences, these sequences form a distinct branch within the clade of group 1 coronavirus RdRp sequences (data not shown). These results suggested that the coronavirus circulating in *M. pusillus* is a novel virus. More interestingly, viral sequences deduced from *Miniopterus magnater* (Fig. 1A, samples 88 and 96) and *Miniopterus schreibersii* (Fig. 1A, sample 86) were also highly similar to those sequences from *M. pusillus* (Fig. 1A), indicating that viruses isolated from these three bat species are the same virus or are of the same lineage.

Attempts to isolate this virus in cell cultures were made. Fecal and respiratory samples were used to infect Madin-Darby canine kidney (MDCK), fetal rhesus kidney (FRhK4) and African green monkey kidney (Vero E6) cells. However, no evidence of virus replication was detected in these cells by cytopathic effect or by RT-PCR.

Characterization of Bat-CoV RdRp, helicase-ExoN, and S sequences. A representative sample from *M. pusillus* (specimen 61 [Fig. 1A]) was selected for further sequence analysis. As mentioned above, our preliminary data indicated that this novel virus is a group 1 coronavirus. On the basis of the conserved regions of group 1 viruses (HCoV-229E, HCoV-NL63, PEDV, TGEV, FIPV, and CCoV), 30 sets of primers were generated for the determination of the viral sequences. Of these PCRs, five additional viral RNA sequences were deduced (Table 2, PCRs 1 and 3 to 6). Alignments of these deduced sequences generated three RNA fragments containing partial sequences for the (i) RdRp gene, (ii) genes C terminal of the helicase gene and N terminal of the hypothetical exonuclease (ExoN) gene, and (iii) S genes (Table 3). The percent sequence identity of each RNA fragment to group 1 to 3 viruses was determined (Table 4). All these RNA sequences shared the highest identity with group 1 coronaviruses (54 to 75%). Phylogenetic analyses of these viral sequences resulted in consensus trees with similar topologies (Fig. 1B, Fig. 3A, and Fig. 4A). In all cases, Bat-CoV clustered with group 1 coronaviruses. In particular, the RdRp and S sequences were most closely related to those of PEDV.

Sequence analysis of the partial sequence of the S gene reveal that this RNA sequence encodes the S2 protein subunit (2, 23). The S protein of coronavirus is known to be heavily glycosylated, and 11 potential N-glycosylation sites were identified (Fig. 2). The deduced sequence contains the HR1 region and part of the sequence of the HR2 region (Fig. 3B and C). These HR regions were separated by an interhelical domain of ~130 amino acid residues (Fig. 2). As with other group 1 coronaviruses, Bat-CoV also has 14 "additional" amino acid residues in both HR regions (Fig. 3C) (2).

The partial RdRp protein sequence of Bat-CoV contains

TABLE 3. Information about Bat-CoV sequences deduced in this study

RNA sequence	Length (nt)[a]	Length of deduced protein sequence (no. of amino acid residues)	Encoding region
1	1,613	537	RdRp
2	591	197	Helicase-ExoN
3	1,448	482	S

[a] nt, nucleotides.

TABLE 4. Nucleotide sequence identities of Bat-CoV RNA fragments to other coronaviruses

RNA fragment	% Identity of RNA fragment to:							
	PEDV	TGEV	HCoV-229E	HCoV-NL63	MHV	HCoV-OC43	SARS-CoV	IBV
RdRp	74	70	75	74	60	61	62	63
Helicase-ExoN	71	67	71	72	55	55	55	55
S	58	54	58	60	40	42	41	43

several conserved motifs of RdRps (motifs A to C, G, and F in Fig. 1A) (18, 25). Motif A has two conserved Asp residues separated by four residues and is known for metal ion binding and for recognition of the ribonucleoside triphosphate sugar ring. Motif B contains the highly conserved Ser, Gly, Thr, and Asn residues and is known to be involved in selection of the correct ribonucleoside triphosphate substrate. Motif C contains the highly conserved SDD sequence and is associated with metal ion and 3′-primer terminus binding. Motif F contains several conserved positively charged basic residues and is associated with nucleoside triphosphate binding. This motif could be divided into three submotifs (F1 to F3) (25). Like other coronaviruses, the F motif of Bat-CoV lacks the F2 submotif. The biological functions of the F2 submotif in other viruses are yet to be determined. The G motif had a conserved SXGXP sequence and is known to be involved in positioning of the 5′ template strand in other RdRps.

The RNA fragment for the helicase-ExoN junction encodes the last 54 amino acid residues of the RNA helicase and the first 143 amino acid residues of the putative exonuclease (Fig. 4B) (21). The partial sequence for the helicase protein contains the conserved helicase motifs V and VI (12). The partial sequence for the putative ExoN protein contains motif 1 of the DEDD exonuclease superfamily (21). At the junction of these two proteins, a conserved cleavage signal for 3CL proteinase was identified (LQS at positions P2 to P1′), which suggests that the junction may be cleaved by 3CL proteinase expressed by Bat-CoV.

DISCUSSION

Interspecies transmissions of animal viruses to humans are permanent threats to human health. The recent transmissions of SARS-CoV (8), West Nile virus (13), Nipah virus (17), and avian influenza virus (15) from animals to humans have highlighted the importance of surveillance of viruses in wildlife. In order to obtain a better understanding of the prevalence of coronaviruses in this geographical region, we collected more than 300 animal samples from 44 animal species. Using a pair of consensus primers for RdRp of coronaviruses, a novel group 1 virus was identified in three *Miniopterus* spp.

Of 12 bat species examined, the novel Bat-CoV was identified in three different bat species from the same genus. Interestingly, *Myotis chinensis* and *Myotis ricketti*, which frequently cohabit with *M. pusillus* (K. Y. Suen, personal communication), were negative in this investigation. These results demonstrate that this virus has a narrow host range. As the viral sequences in *Miniopterus* spp. are highly similar, our data imply that there are frequent interspecies transmissions between these species. It is not certain which *Miniopterus* species is the natural host of the virus. However, the majority of *M. pusillus* bats were found to be infected by this virus. In addition, the detection rates of this virus in *M. pusillus* in the summer of 2003 and 2004 were similar (57% in 2003 and 67% in 2004). These observations suggest that *M. pusillus* is likely to be the major reservoir of this virus. As the majority of *M. pusillus* bats in Hong Kong are known to migrate to tropical areas to overwinter (K. Y. Suen, personal communication), we do not know the prevalence of Bat-CoV in this species in winter. Further work on the ecology and behavior of these three bat species in Hong Kong is required to better understand the dynamic of this virus in these animals.

Although all of the infected bats were shown to be healthy upon physical examination, it is not known whether this novel virus is pathogenic in bats. Both fecal and respiratory samples were positive for the virus. However, more than 50% of fecal samples from *M. pusillus* contained this novel virus compared to 26% of respiratory specimens from the same bats, implying that this virus may have a predominantly enteric tropism. Further work is required to elucidate the persistence of the infection, tissue tropism, and possible pathogenicity of this virus.

The deduced Bat-CoV sequences have the typical features of coronaviruses. The virus has the highest sequence identity to group 1 coronaviruses but is clearly distinct from previously known group 1 viruses. Our phylogenic analyses of these viral sequences also suggest that the virus is a group 1 virus. These findings are further supported by the fact that the S protein of Bat-CoV contains the unique signature of group 1 coronaviruses (i.e., the unique 14 amino acids in HR1 and HR2) (2). Recombination is common in coronaviruses and is thought to contribute to the emergence of new coronaviruses (9). Our limited sequencing results do not allow us to draw any conclusions of the origin of this virus. However, the low sequence homology between Bat-CoV sequences and other coronavirus sequences at least suggest that this novel virus is not a recent recombinant from existing coronaviruses. We are currently attempting to sequence the rest of the viral genome for a full sequence characterization of this virus. However, these efforts are hampered by the inability to culture the virus in vitro.

Apart from the Bat-CoV, we did not identify other coronaviruses in our samples. However, one should note that our results could not reveal a complete picture of the prevalence of coronaviruses in this geographical region. First, our test relies on the detection of viral sequence. Animals with a past coronavirus infection would be negative in our assay. Second, due to the limited sample sizes of each animal species, we might miss the viruses which are circulating in low frequency. Besides, the conserved primers used in this study were based on

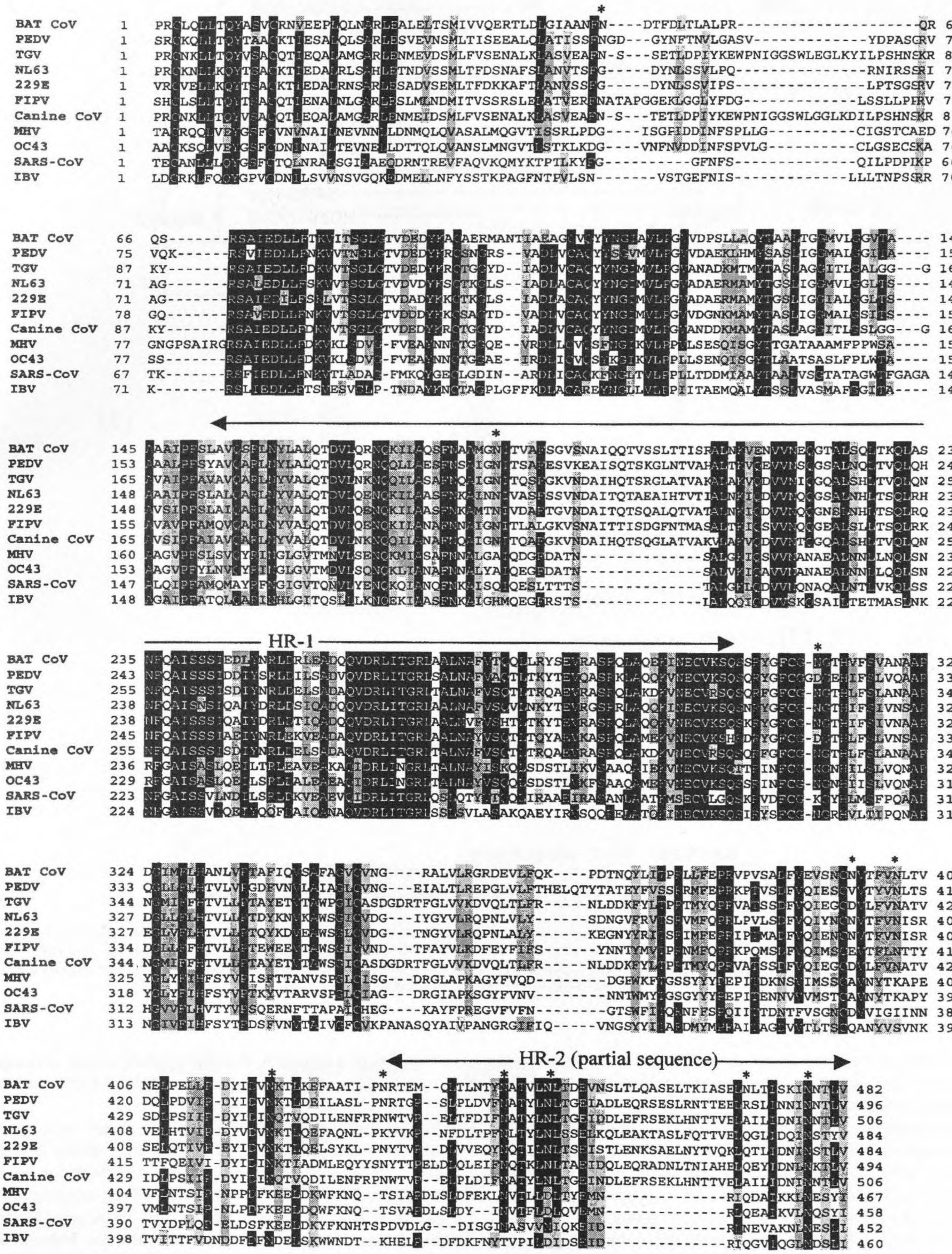
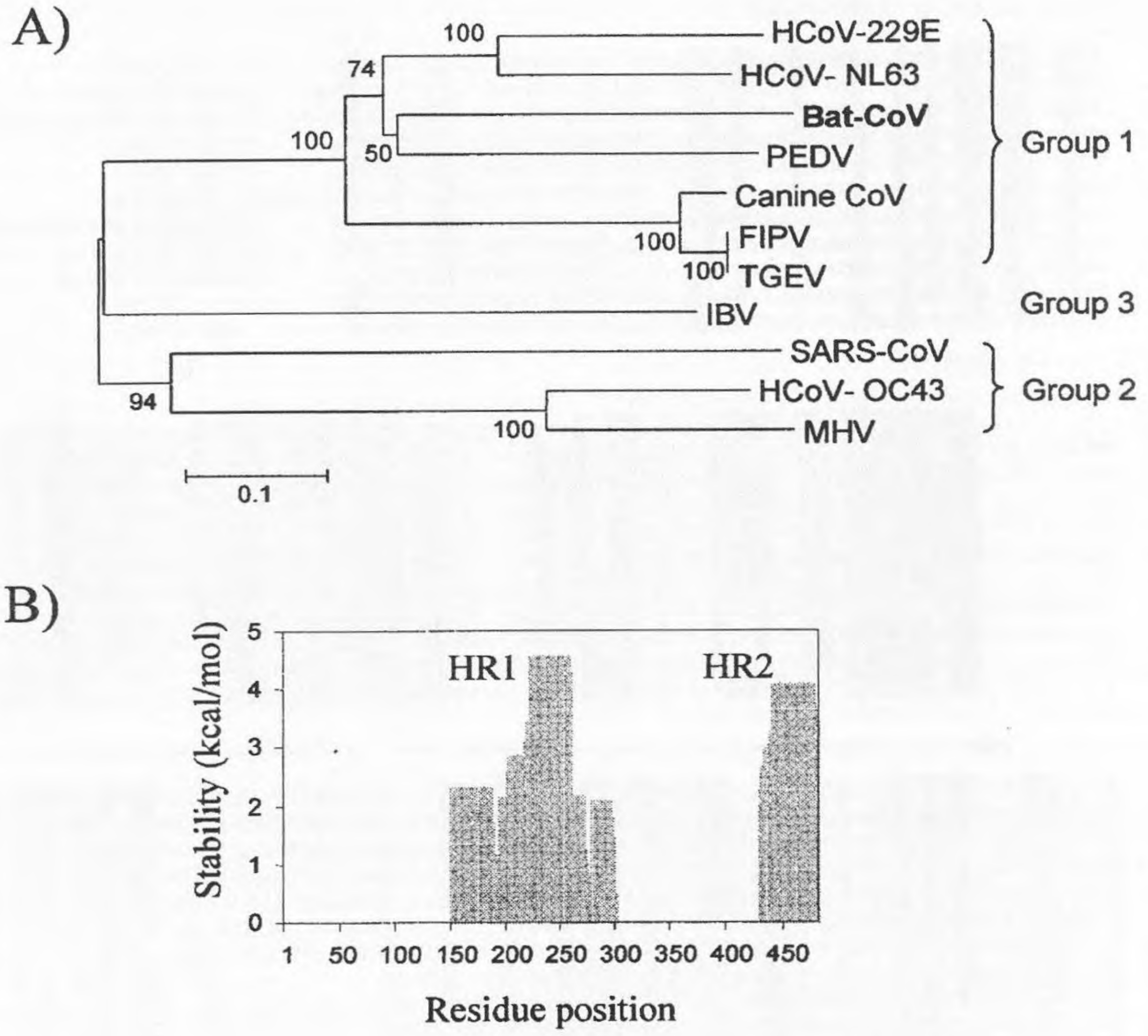


FIG. 2. Protein sequence alignment of coronavirus S proteins (partial sequence). The HR1 and HR2 regions are indicated. The locations of potential N-glycosylation sites in the Bat-CoV sequence are marked by asterisks. TGV, TGEV; NL63, HCoV-N63; 229E, HCoV-229E; OC43, HCoV-OC43.



C)

```
HR1 sequence:

  d   a  d   a  d   a  d   a  d   a   a  d   a  d
LAVQSRLNYLALQTDVLQRNQKILAQSFNAAMGNITVAFSGVSNAIQQTV
  a  d   a  d   a   a  d   a  d   a  d   a  d   a
SSLTTISRALNKVENVVNEQGTALSQLTKQLASNFQAISSSIEDLYNRLD
 d   a  d   a  d      a  d   a  d   a  d   a  d
RLEADQQVDRLITGRLAALNAFVTQQLLRYSEVRASRQLAQEKINECVK


Partial HR2 sequence:
 a  d   a  d   a  d   a  d   a  d   a  d   a  d   a
NRTEMQLTLNTYNATVLNLTDEVNSLTLQASELTKIASELNLTISKINNTLV
```

FIG. 3. (A) Phylogenetic analysis of RNA sequences for the S gene (partial sequence). (B) Predicted coiled-coil regions in the deduced S-protein sequence. The coiled-coil regions were predicted by Stablecoil 1.0 with a 35-residue window width. The HR1 and HR2 regions are indicated. (C) HR1 and HR2 in the S protein of Bat-CoV. The a and d positions of the strongest predicted coiled-coil heptad repeats are indicated. The 14-amino-acid residue insertions that are unique in group 1 viruses are underlined.

available coronavirus sequences, and these primers might not be able to detect coronaviruses that are genetically more divergent from previously known coronaviruses.

We previously reported that SARS-CoV could be isolated from Himalayan palm civets (8). It should be noted that the SARS-CoV-positive animals in our previous study were obtained from wild animal markets and not captured in the wild. None of the civets examined in our current study ($n = 21$) was positive for SARS-CoV by both serological and molecular tests. The results from our present study do not exclude the possibility that the civet is the natural host of SARS-CoV, but our results at least indicate that SARS-CoV is not broadly circulating in wild civets. Further investigations are required to elucidate the natural reservoir of SARS-CoV, especially in mainland China.

In conclusion, we reported that a novel coronavirus was identified from bats. The virus has the highest homology to group 1 coronaviruses. Bats are the reservoir for lyssaviruses and henipaviruses and are responsible for emerging diseases in humans. It is not known whether this virus would cause zoonotic disease in humans or other animals. Further investigations are needed to understand the ecology and pathogenicity of this virus. In addition, this study also highlighted our poor understanding of viruses in wild animals. Given the

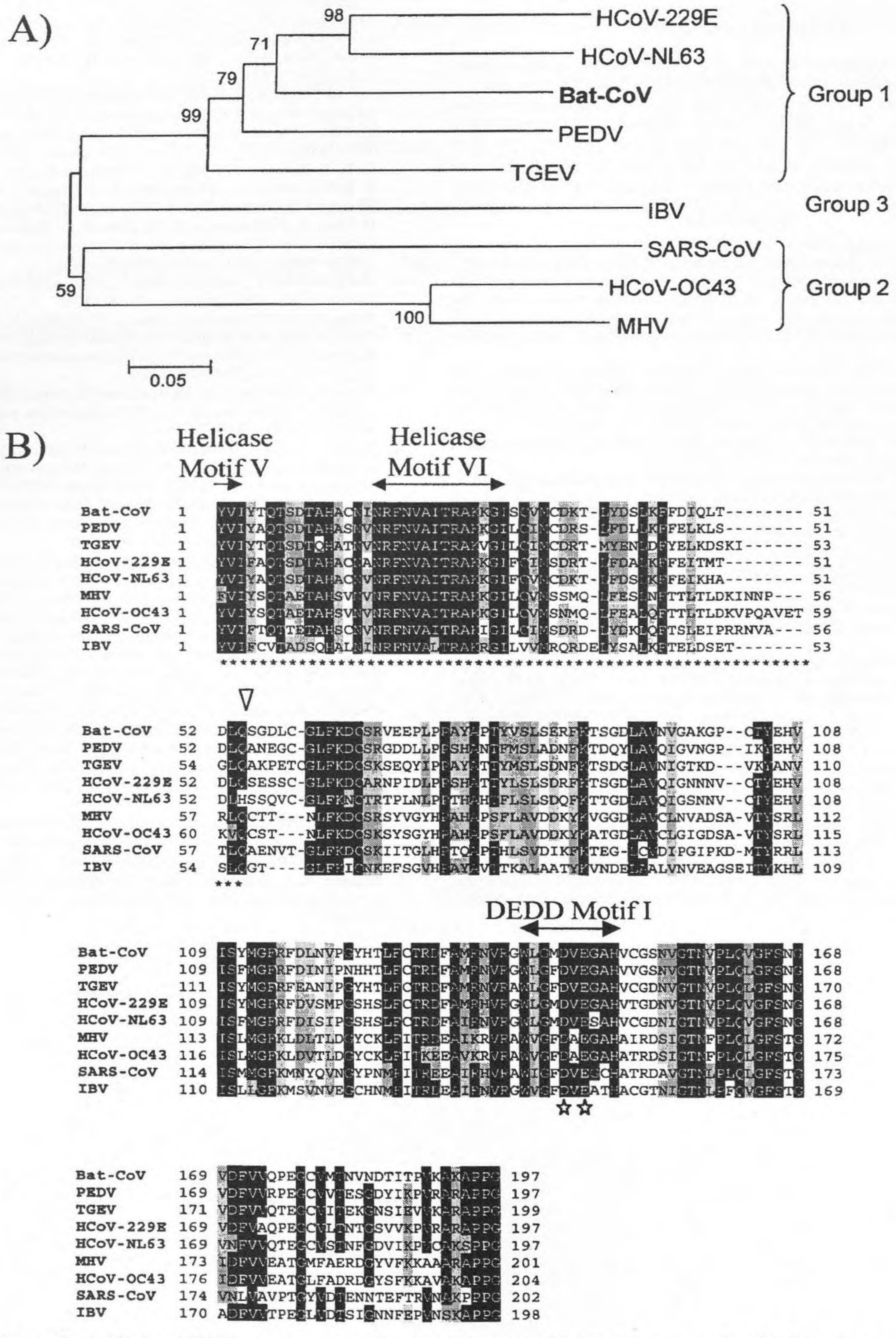


FIG. 4. (A) Phylogenetic analysis of RNA sequences coding for helicase-ExoN (partial sequence). (B) Protein sequence alignment of coronavirus helicase-ExoN. The conserved motifs for helicases and the first motif for ExoN (DEDD motif I) are indicated. The invariant acidic residues in DEDD motif 1 are labeled with white stars below the sequences. The inverted open triangle above the sequences marks the predicted 3CL proteinase cleavage site.

catastrophic consequences of SARS, further surveillance work on viruses in wildlife should be encouraged.

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