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► **To cite this version:**

Channa Mey, Artem Metlin, Veasna Duong, Sivuth Ong, Sotheary In, et al.. Evidence of two distinct phylogenetic lineages of dog rabies virus circulating in Cambodia.. *Infection, Genetics and Evolution*, 2016, 38, pp.55-61. 10.1016/j.meegid.2015.12.011 . pasteur-01429885

HAL Id: pasteur-01429885

<https://pasteur.hal.science/pasteur-01429885>

Submitted on 9 Jan 2017

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TITLE: Evidence of two distinct phylogenetic lineages of dog rabies virus circulating in Cambodia

SHORT TITLE: Phylogeny of rabies virus in Cambodia

CONTENTS CATEGORY: Standard (full-length) paper

AUTHORS: Channa Mey^{1#}, Artem Metlin^{1#}, Veasna Duong¹, Sivuth Ong¹, Sotheary In², Paul F. Horwood¹, Jean-Marc Reynes³, Hervé Bourhy⁴, Arnaud Tarantola⁵, Philippe Buchy^{1,6*}

¹Virology Unit, Institut Pasteur in Cambodia

²Rabies Prevention Center, Institut Pasteur in Cambodia

³Unité de Biologie des Infections Virales Emergentes, Institut Pasteur. Centre International de Recherche en Infectiologie, Lyon, France

⁴Institut Pasteur, Unit Lyssavirus Dynamics and Host Adaptation, WHO Collaborative Centre for Reference and Research on Rabies, Paris, France

⁵Epidemiology and Public Health Unit, Institut Pasteur in Cambodia

⁶GlaxoSmithKline Vaccines R&D, 150 Beach Road, Singapore

*Corresponding author

[#]these authors contributed equally

HOST LABORATORY: Virology Unit, Institut Pasteur in Cambodia 5, Monivong Blvd - PO Box 983 Phnom Penh, Cambodia.

CORRESPONDING AUTHOR'S ADDRESS: Institut Pasteur in Cambodia, Virology Unit, 5 Monivong blvd, Phnom Penh, Cambodia - GlaxoSmithKline, Vaccines R&D, 150 Beach Road, Singapore. Email: buchyphilippe@hotmail.com

Summary

This first extensive retrospective study of the molecular epidemiology of dog rabies in Cambodia included 149 rabies virus (RABV) entire nucleoprotein sequences obtained from 1998-2011. The sequences were analyzed in conjunction with RABVs from other Asian countries. Phylogenetic reconstruction confirmed the South-East Asian phylogenetic clade comprising viruses from Cambodia, Vietnam, Thailand, Laos and Myanmar. The present study represents the first attempt to classify the phylogenetic lineages inside this clade, resulting in the confirmation that all the Cambodian viruses belonged to the South-East Asian (SEA) clade. Three distinct phylogenetic lineages in the region were established with the majority of viruses from Cambodia closely related to viruses from Thailand, Laos and Vietnam, forming the geographically widespread phylogenetic lineage SEA1. A South-East Asian lineage SEA2 comprised two viruses from Cambodia was identified, which shared a common ancestor with RABVs originating from Laos. Viruses from Myanmar formed separate phylogenetic lineages within the major SEA clade. Bayesian molecular clock analysis suggested that the time to most recent common ancestor (TMRCA) of all Cambodian RABVs dated to around 1950. The TMRCA of the Cambodian SEA1 lineage was around 1964 and that of the SEA2 lineage was around 1953. The results identified three phylogenetically distinct and geographically separated lineages inside the earlier identified major SEA clade, covering at least five countries in the region. A greater understanding of the molecular epidemiology of rabies in South-East Asia is an important step to monitor progress on the efforts to control canine rabies in the region.

Highlights:

We performed the first extensive rabies virus evolutionary study in Cambodia;

We generated and analyzed 149 Cambodian rabies virus nucleoprotein sequences;

We identified 3 phylogenetic lineages in the major South-East Asian clade.

Key words: rabies virus evolution, rabies epidemiology, canine rabies, Cambodia, South-East Asia

1. Introduction

The developing world is severely affected by rabies, causing deaths in humans as well as in animals and resulting in significant economic losses. Indeed, it is estimated that globally canine rabies causes approximately 59,000 human deaths, over 3.7 million disability-adjusted life years (DALYs) and 8.6 billion USD in economic losses annually (Hampson et al., 2015).

Rabies virus (RABV) belongs to the family *Rhabdoviridae* of the order *Mononegavirales* which comprises at least 10 genera (<http://www.ictvonline.org>). The *Lyssavirus* genus includes classical RABVs, found in a wide range of different animal species throughout the world. All 13 other species of *Lyssavirus* except two have been isolated from bats, originating from different locations in Europe, Asia, Africa and Australia. RABV has a negative-sense RNA genome of about 12 kb encoding five proteins (Tordo et al., 1986, 1993). The nucleoprotein gene (N-gene) is widely used for molecular characterization and phylogenetic analysis of RABVs (Kissi et al., 1995). The large number of N-gene sequences published makes this an obvious region of the genome to study (Johnson et al., 2002, Chupin et al., 2013). The N-gene is also suitable for estimations of evolutionary rate and subsequent analysis of evolutionary history (Holmes et al., 2002; Bourhy et al., 2008).

Most South-East Asian countries are affected by rabies, but with the exception of a few countries, there is limited accurate epidemiological information available. According to the World Organization for Animal Health (OIE), there were 15 animal rabies outbreaks recorded in Vietnam in 2012 with 86 rabies cases found in dogs in five different administrative divisions of the country. For Laos it was only mentioned that rabies was present in 2012 with no indication of the number of outbreaks/cases. In Myanmar, the number of human rabies cases per year was estimated at about 1000 while in Thailand it was less than 25 (Gongal & Wright, 2011).

The only reliable data on human and animal rabies in Cambodia are available from the Institut Pasteur in Cambodia (IPC) where rabies diagnosis activity has been performed routinely since 1998, but laboratory-confirmed cases of rabies in dogs were recorded since the 1970s (Reynes et al., 1999). In the second half of the 1970s, dogs which are the most important vector for human rabies in Asia (Dodet et al., 2001) almost disappeared from Cambodia because of starvation. However, dog numbers rebounded in subsequent decades and rabies consequently became a serious public health concern. During the mid-1980s, the number of patients who have sought medical assistance after a dog bite has been at least 4000 per year, but the number of reported rabies deaths has been very low, partly because of improved post-exposure treatment delivery. Deaths occurring at home are usually not reported to health services. From

1982 to 1991 a total of 5437 animal bites were recorded and 51 human rabies deaths were reported (WHO/Rab. Res./93.44). Since 1994, rabies stopped being a notifiable disease in Cambodia. Thus, the only data available after 1994 is from the IPC, which established a rabies post-exposure treatment centre in 1995 (Reynes et al., 1999). From mid-1995 to 2007, 149,224 post-exposure prophylaxis (PEP) treatments were provided at the IPC Rabies Prevention Centre (Reynes, 1999; Ly, 2009; WHO/EMC/ZOO/96.8) and still 67 fatal human cases were reported countrywide following dog bites.

During 1998-2007, 610 animal samples (49% of all specimens tested) originating mostly from dogs with suspected rabies were found rabies-positive (Ly et al., 2009). A predictive model established by Ly et al. (2009) estimated that 810 human rabies cases occurred in 2007 alone. In 2008-2013, rabies was recorded mostly in dogs (n=932, 99.3%). Rabies cases in cats (n=3, 0.3%), bovines (n=3, 0.3%) and swine (n=1, 0.1%) were detected in very rare occasions (Institut Pasteur in Cambodia, unpublished data).

At present, the dog population in Cambodia is estimated to exceed 5 million animals and nearly one-third of the 250 Cambodian households surveyed in a preliminary study could recall at least one dog bite experience during 2004–2009 (Institut Pasteur in Cambodia, unpublished). Overall, the risk proportion (total number dog bites/total human population) in Cambodia was 5.6% (75/1339). This equates to an annual incidence of 1120 dog bites per 100 000 people (Lunney et al., 2012). Other mammals like mongoose, bats or other wild animals are sometimes suspected in Asia to contribute to maintaining the circulation of rabies. In Cambodia, Reynes et al. (2004) found serological evidence of Lyssavirus infection in bats. Several studies on RABV molecular epidemiology in Asia (Bourhy et al., 2008), namely in Vietnam (Nguyen et al., 2011; Yamagata et al., 2007), Thailand (Ito et al., 1999) and Laos (Ahmed et al., 2015) were conducted previously.

The objective of the present study was to perform a retrospective molecular and phylogenetic characterization of RABVs from dogs originating from different parts of Cambodia, to compare them to strains originating from other South-East Asian countries and to analyze the circulation of dog RABVs in this region.

2. Materials and Methods

2.1. *Samples and Viruses.* Dog brain samples received by the IPC Virology Unit from different regions of Cambodia between 1998 and 2011 were included in the study. In total, 149 rabies-positive samples obtained from dogs originating from 20 administrative regions of Cambodia were selected by location and year of sampling (Supplementary Table 1). Dog's heads were usually referred to the IPC lab

from people who were consulting for PEP following the animal bite. The animal samples were tested by a standard direct fluorescent antibody test (FAT) (Dean et al., 1996) using an anti-rabies nucleocapsid conjugate (Anti-Rabies Nucleocapsid Conjugate; lyophilized, adsorbed #357-2112, Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions.

2.2. *RNA purification, Reverse Transcriptase PCR and Nucleotide Sequencing.* The diagnostic method for the initial detection of the RABV from clinical samples used a hemi-nested RT-PCR targeting a conserved region of the L-gene to confirm results of FAT as previously published (Dacheux et al., 2008). The target size of amplicons obtained after the second round of PCR was approximately 250 base pairs. Negative and positive control samples were included successively for each extraction, reverse transcription, and for all PCR steps.

To generate amplicons for sequence analysis, two overlapping RT-PCRs were conducted targeting the N-gene (Yang et al., 2011), which has been extensively used for phylogenetic analyses of RABVs. The two sequencing PCRs (Table 1) were conducted using the same reaction conditions and cycling protocols as previously described (Dacheux et al., 2008). The first PCR, using the primers RVN-71F (5'-ATGGATGCCGACAAGATTGTATTC-3') and RVN-1118R (5'-GAATTCCTCTCCCAGATAGCC-3') generated a 1048 bp amplicon; the second PCR, using the primers RVN-1091F (5'-CTAGGGGGCTATCTGGGAGA-3') and RVN-1562R (5'-CGGCCAGACCGGCTCTAACAC-3') produced a 472 bp amplicon. The PCR products were sequenced by Sanger method at a commercial facility (Macrogen, Seoul, South Korea) and resulted in a combined N-gene sequence of 1492 nucleotides.

Phylogenetic Analysis. The raw sequences were edited with CLC Genomic Workbench Version 3.6.1. Multiple sequence alignment was built with Clustal Omega tool and edited with Jalview package Version 2.8. (Waterhouse et al., 2009) and GeneDoc Version 2.6.002 (Nicholas et al., 1997). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Entire and partial (positions 56 to 454 according to ERA vaccine strain, GenBank #EF206707) N-gene sequences of RABVs from Cambodia, Vietnam, China, Thailand, Laos and Myanmar as well as a rabies vaccine strain PV (an outgroup) were included in the study (Supplementary Table 2). To choose the most suitable model for phylogeny, a Model Selection analysis was performed with MEGA6 package (Nei & Kumar, 2000; Tamura et al., 2013).

Phylogenetic analyses were conducted on RABV sequences including selected sequences sourced from the GenBank Database. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1346 positions in the final dataset for entire N-gene sequences, and 381 positions in the final dataset for partial N-gene sequences. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

The evolutionary history of the entire N-gene sequences was inferred using the Maximum Likelihood method. The evolutionary distances were computed using the Tamura 3-parameter model (Tamura, 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3114)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bootstrap test of phylogeny with 1000 replicates was performed. The analysis involved 192 nucleotide sequences, obtained in the present study (Supplementary Table 1) and retrieved from public databases (Supplementary Table 2).

For the partial N-gene, sequences were analyzed as above with some modifications. The evolutionary history was inferred using the Minimum Evolution (ME) method (Rzhetsky & Nei, 1992). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Nei & Kumar, 2000) and are in the units of the number of base differences per sequence. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-Joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree.

2.3. *Evolutionary analysis.* To determine the comparative evolutionary dynamics of RABVs from Cambodia, we used the BEAST package (Drummond and Rambaut, 2007) to estimate the rate of nucleotide substitution per site, the Time to Most Recent Common Ancestor (TMRCA), and the Maximum Clade Credibility (MCC) tree of the complete coding region of N-gene sequences (Drummond and Rambaut, 2007) utilizing information on the exact year of sample collection. This analysis used the GTR model of nucleotide substitution as well as a different substitution rate for each codon position. A Bayesian Skyline Plot (BSP) was used as a coalescent prior. This provides a piecewise graphical

depiction of changes of relative genetic diversity through time ($N_e\tau$), where N_e is the effective population size and τ is the host-to-host generation time. We also utilized both strict and relaxed (uncorrelated lognormal) molecular clocks. A previous analysis of RABV evolution found that the uncorrelated lognormal relaxed clock and constant population size model provided a better fit to the data (Bourhy et al., 2008), and this was confirmed in a preliminary analysis of our data, although the parameter values estimated were similar under both models. The degree of uncertainty in each parameter estimate is provided by the 95% highest posterior density (HPD) values, while posterior probability values provide an assessment of the degree of support for each node on the tree. All Markov Chain Monte Carlo chains were run for sufficient time at 200 million steps to ensure statistical convergence, with 10% removed as burn-in.

3. Results

3.1. Sampling. The geographical origins of the Cambodian dog samples analyzed in this study are shown in Figure 1. As there was no information on the exact location where the bite occurred, the cases were attached to the local district health center, which in Cambodia is usually located no more than 5 kilometers from the furthest point of the district.

3.2. Phylogenetic analyses. The complete N-gene sequences of 149 RABV isolates collected in 20 provinces of Cambodia were generated and submitted to GenBank (Supplementary Table 1).

The Model Selection analysis with MEGA6 package revealed that for the dataset of entire and partial nucleoprotein sequences included in the present study, the Tamura 3 – parameter model with a discrete Gamma distribution (+G) had the lowest Bayesian Information Criterion (22395.836 for entire and 10845.81921 for partial nucleoprotein sequences) and was considered to describe the substitution pattern the best.

The complete N-gene sequences (1492 nucleotides in length, positions 71 to 1562, cut with the GeneDoc package to the length of 1346 nt to fit the equal size of the sequences of the reference strains, taken from the public databases) were used to build a Maximum Likelihood tree (Fig. 2).

Phylogenetic reconstruction identified the South-East Asian (SEA) clade that included all the nucleoprotein gene sequences of RABVs from Cambodia, Vietnam, Thailand, Laos and Myanmar. Inside the SEA clade 3 distinct phylogenetic lineages (SEA1, SEA2 and SEA3) were distinguished based on the bootstrap values obtained for the ancestral nodes of each cluster. Most of the viruses from Cambodia

were closely related to viruses from Thailand, Vietnam and Laos and formed the lineage SEA1. We identified three putative sub-lineages within the SEA1 lineage, and two of them were geographically restricted to Thailand and Laos (putative sub-lineages 2 and 3, respectively). The SEA2 lineage was comprised of two viruses from Cambodia and a group of RABVs from Laos. One of these Cambodian RABVs was isolated from a dog in Phnom Penh in 2005; the second was isolated from a dog in 1997 from Banlung, the capital city of the Ratanakiri province in the North-East part of Cambodia, a region that shares common borders with Laos and Vietnam. This strain was already previously described by Bourhy et al. (2008). The two Cambodian SEA2 strains shared a common ancestor with RABVs isolated in Laos in 1999-2002 (Bourhy et al., 2008) and in 2011-2012 (Ahmed et al., 2015). Viruses from Myanmar formed a separate phylogenetic lineage SEA3.

Phylogenetic analysis was then conducted on 194 partial N-gene sequences generated in the present study and other strains originating from South-East Asian countries (Supplementary Table 2). Rabies virus 9337SWI isolated from a bat in 1993 (EBLV2) was used as an out-group to build this tree. This analysis allowed a comparison with the shorter sequences obtained by Nguyen et al. (2011) from Vietnamese RABVs, resulting in further support for the clade and lineage groupings (Supplementary Fig. 1). The SEA1 lineage, which was initially proposed by Yamagata et al. (2007), was comprised of viruses from Cambodia, Thailand, Laos and Vietnam and corresponded to the subgroup 1a suggested by Nguyen et al. (2011); whereas the SEA2 and SEA3 lineages established in the present study were distinct from the subgroup 1a and 1b, but still contained within the broader Group 1 described previously by Nguyen et al. (2011).

3.3. *Evolutionary analysis.* Bayesian molecular clock analysis performed on complete N-gene sequences suggested that the TMRCA of all 155 Cambodian rabies viruses dated back to 1950 (95% HPDs: 1935 to 1961; Fig. 3). The TMRCA of the Cambodian SEA1 lineage was around 1964 (95% HPDs: 1955 to 1971) and that of SEA2 lineage was around 1953 (95% HPDs: 1938 to 1969). The nucleotide substitution rate was estimated at 3.13×10^{-4} substitutions/site/year (s/s/y; 95% HPD: $2.46 \times 10^{-4} - 3.79 \times 10^{-4}$). Using Bayesian skyline plots as an inference tool, the patterns of relative genetic diversity in Cambodian rabies virus increased to its maximum approximately in 2005 followed by a small decline afterward.

4. Discussion

Domestic/stray dog rabies is still widespread throughout the world, occurring in over 80 countries/territories (predominantly in the developing world) and is implicated in more than 99% of all cases of human rabies (WHO, 2012). The present study aimed to provide greater clarity on dog rabies molecular epidemiology in South-East Asian countries using the sequences generated here in addition to the limited number of sequence data publicly available. More specific attention was paid to Cambodia, which is severely affected by rabies but where the epidemiology of the disease remains unclear. Currently, there is no active rabies surveillance system in Cambodia, and the existing data is only available through the rabies diagnosis efforts and the PEP treatment activities of the IPC. As such, reliable analysis of the precise dynamics of dog rabies in Cambodia by year cannot be performed, but the available data confirms that the disease exists and is widespread throughout the country.

Previously a large-scale research study covering South-East Asian countries provided the first definitive evidence for a widely distributed Asian clade comprising viruses from Myanmar, Thailand, Laos, Cambodia, Vietnam and some other countries, but the lineages in which the strains were clustering could not be determined (Bourhy et al., 2008). Ito et al. (1999) performed the first detailed study of rabies molecular epidemiology in Thailand in which six lineages of RABV were identified. At least four of them were endemic to Central (T1C), Southern (T2S) and Northern (T1N1 and T1N2) areas of Thailand whereas the two other lineages (T1CS and T2NC) overlapped different areas. Yamagata et al. (2007) proposed the SEA1 lineage of RABV including viruses from Thailand and Vietnam. Later Nguyen et al. (2011) established the existence of two major RABV lineages in Vietnam (Groups 1 and 2), with Group 1 further subdivided into two subgroups (1A and 1B). Subgroup 1A represented the SEA1 lineage previously described by Yamagata et al. (2007). Subgroup 1B was comprised of viruses from Vietnam, China and Philippines; and Group 2 included viruses from China and Vietnam. Ahmed et al. (2015) summarized that RABVs from Thailand, Malaysia, Cambodia, Laos and Vietnam originated from a common ancestor; and the lineages circulating in Laos were closely related to each other but were not the same as in other South-East Asian countries.

In this first extensive study of RABV molecular epidemiology in Cambodia, a large pool of dog RABV nucleoprotein gene sequences were generated from strains detected in 20 provinces of Cambodia between 1998 and 2011. Figure 1 shows that these viruses were nevertheless mostly collected in the capital and its surrounding provinces. This is because the only rabies diagnostic facility – the Rabies National Reference Centre (IPC, Virology Unit) – and the major post-exposure treatment facility (IPC, Rabies Vaccination Centre) are both located in Phnom Penh. As a result, the samples collected from

suspected rabid dogs were rarely received from the districts that are very distant from the capital. Nevertheless, IPC's Rabies Reference Centre adequately covers the South-East region of Cambodia, which includes approximately 60-70% of Cambodian's human and canine populations. Cambodian viruses were included for phylogenetic analysis with RABV sequences from China, Laos, Myanmar, Thailand and Vietnam. Viruses originating from dogs were given preference to be included in analyses. Some human RABV sequences from Vietnam were also included because of the lack of suitable representative sequences from dog origin from this country. However, as dogs are the major rabies reservoir in Asia (Dodet et al., 2001), and most human cases had a history of a dog bite (Nguyen et al., 2011), this should not have introduced a significant bias into the analysis.

The results presented in this study show that the major SEA phylogenetic clade covers at least five countries in the South-East Asian region, including Cambodia, Vietnam, Thailand, Laos and Myanmar. This data, together with the timescale of RABV evolution, are in agreement with the results published earlier by Bourhy et al. (2008). In the present study, we attempted to investigate the presence of distinct phylogenetic lineages inside this large clade.

Most of the lineages identified inside the SEA clade were associated with their geographical origins. All of the viruses from Cambodia, except two (EU086170: Bourhy et al., 2008; and P0709509: this study), clustered with RABVs from Thailand, Vietnam and Laos within the SEA1 lineage, which appears to be the most widespread lineage throughout South-East Asia. We propose the existence of at least 3 putative sub-lineages inside the SEA1 lineage. The sub-lineage 1 comprised RABVs from Cambodia, Thailand, Vietnam and Laos. Two dog RABVs (GQ303556 & JN786877) clearly formed a distinct sub-lineage 2. Failure of rabies PEP associated with unusual manifestation of the disease was previously described with the strain GQ303556 (Shantavasinkul et al., 2010). The second virus, JN786877, after a series of passages in BHK cells rapidly killed mice inoculated by intramuscular route (Virojanapirom et al., 2012). Although only a small number of viruses from this phylogroup have been described, the potential association of these strains with an increased virulence should be further explored. Sub-lineage 3 comprised RABVs isolated in Laos (2011-2012) described by Ahmed et al. (2015). The SEA1 lineage correlates with the group 1/subgroup 1a, determined earlier by Nguyen et al. (2011) and the South-East Asian lineage 1 identified by Yamagata et al. (2007).

The SEA2 lineage seemed to be distributed in Cambodia and Laos, whereas the distribution of the SEA3 lineage appeared to be limited to Myanmar. The newly established SEA2 lineage comprised several RABVs from Laos and only two Cambodian strains: one from Phnom Penh and one from

Banlung, located in the North-East of Cambodia near the Laos border. Taking into account that Laos RABVs included in the SEA2 lineage were either from the Vientiane capital, or from the Champasak province which is bordering Cambodia, the circulation of this lineage in Northern Cambodia and Southern Laos with occasional spread to the capitals of the two countries seems plausible. Further studies need to be conducted to confirm the precise origin and distribution of this lineage as there is a clear bias of recruitment of samples towards the capital cities. Only few strains originating from provinces bordering Laos and Vietnam were available for our study: one from the province of Preah Vihear, detected in 2009, and one from Banlung, Ratanakiri province, detected in 2010. It was unfortunately not possible to find any strains from the northern provinces of Cambodia, close to Laos, and thus interpretations must be exercised with caution. In Myanmar, only one lineage of RABVs was identified, comprising the SEA3 group. However, only three RABV sequences from Myanmar were available for analysis, all originating from 1999, so additional studies with more RABV sequences from this country are needed to confirm this observation.

The SEA1 lineage was previously identified by Yamagata et al. (2007) although the bootstrap values were very low (32-40). It comprised viruses from Thailand and Vietnam and the authors proposed that because Cambodia and Laos are situated between Vietnam and Thailand, there was a possibility that RABVs from Cambodia and Laos were genetically related to the SEA1 lineage. This hypothesis is now confirmed by the present study. Such a wide distribution suggests the presence of suitable conditions allowing the intensive circulation of the viruses belonging to that lineage in the dog populations of Thailand, Laos, Cambodia and Vietnam. Illegal trade of stray dogs, essentially for meat consumption, between these four countries could well have contributed to the regional spread of RABVs from the SEA1 lineage (<http://www.animals24-7.org/2014/06/08/dog-meat-traffic-still-spreads-rabies-in-vietnam/>; Ahmed et al., 2015).

The evolutionary analysis of the data generated in this study resulted in a comparative evolutionary dynamic analysis of the Cambodian RABVs including the estimation of the rate of nucleotide substitution per site, the TMRCA, and the MCC analysis of the complete coding region of N-gene sequences. Our analysis suggests that the SEA1 lineage had a most recent ancestor in common with the newly established SEA2 lineage. The two lineages apparently diverged around 1950. Interestingly, the SEA2 lineage shared a common ancestor with the Lao viruses around 1953, suggesting that either this lineage was first introduced to Laos from Cambodia and then was largely outcompeted in Cambodia by the SEA1 lineage, or that sporadic introductions of the SEA2 RABVs took place from Laos to

Cambodia during the 1960s. It is possible that this lineage is more widespread in Cambodia than it appears, but additional studies are required to confirm this hypothesis. During the conflict period of the Khmer Rouge regime, stray dogs almost disappeared due to starvation and human consumption, which could also have impacted the circulation of the RABV lineages in the country. Based on the limited number of sequences available for this analysis, we established that viruses from Myanmar diverged from the other viruses of the major SEA clade approximately in 1942. Interestingly, the viruses of Chinese origin included in this study had a TMRCA around 1734 and since then seem to have evolved independently from the SEA clade.

The present study identified three phylogenetically distinct lineages inside the major South-East Asian phylogenetic clade, with distributions representatives from the five countries in the region. Because the SEA1 lineage was the most widespread in Cambodia, the detection of a new SEA2 lineage in the country could have resulted either from a recent introduction of RABVs from abroad (e.g. Laos), or from the identification of a group of less prevalent viruses. Further molecular and epidemiological investigations are required to answer this question. To elucidate the distribution of the local lineages of the SEA clade and their roles in the molecular epidemiology of rabies, namely the dynamics and spread of rabies in South-East Asia, further studies need to be conducted on an international level. To better define the molecular characteristics of the SEA clade and achieve a more unified terminology, more Asian RABV sequences should be generated, eventually through a regional initiative. These analyses should also include rabies viruses from wildlife and bat *Lyssaviruses* of South-East Asian origin, the existence of which was suggested based on serological evidence (Reynes et al., 2004).

The wide distribution of the SEA1 lineage across SE Asia suggests a relatively frequent mixing of dog populations throughout the region. This may provide new insights to assist the human and animal health authorities in these countries to take suitable actions to reduce dog circulation in their efforts to eradicate rabies. The Asian Rabies Elimination Strategy (ASEAN, 2015) has outlined the ambitious target of eliminating canine rabies in South-East Asia by 2020. Further clarity on the circulation of RABVs and dog populations will be important information to inform dog population management strategies.

5. Acknowledgments

The authors would like to thank staff of the IPC Rabies Centre and International Centre for Vaccinations and the staff of the IPC Virology Unit.

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Figure 1. Geographical origins of the Cambodian rabies viruses sequenced for the purpose of the present study. The map was built with ArcMap 10 package (ESRI, Redlands, CA, USA).

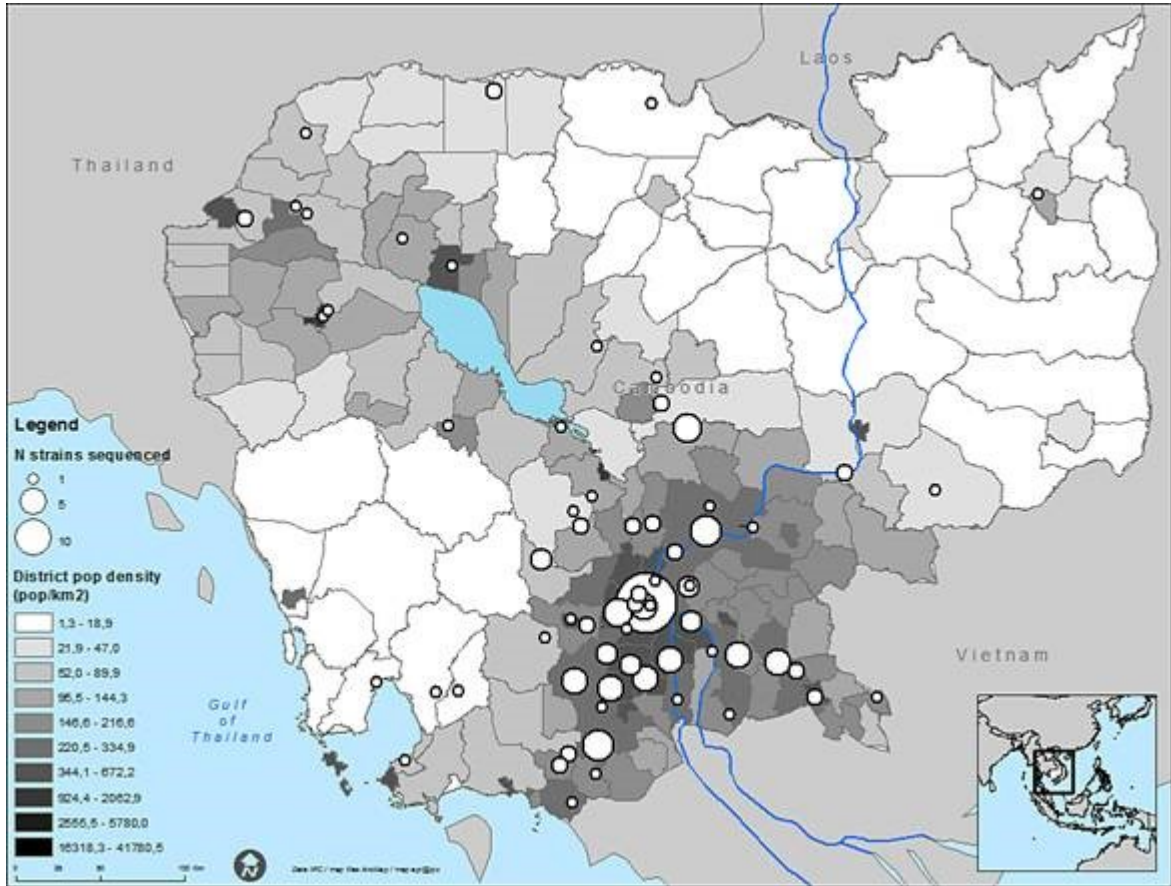


Figure 2. Evolutionary relationships of South-East Asian rabies viruses based on complete N-gene sequences. The tree was built using Maximum Likelihood method based on Tamura 3-parameter method (Tamura, 1992) to compute the evolutionary distances. The tree with the highest log likelihood (-7633.4158) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 192 nucleotide sequences. There were a total of 1346 positions in the final dataset. Bootstrap values lower than 70 are hidden.

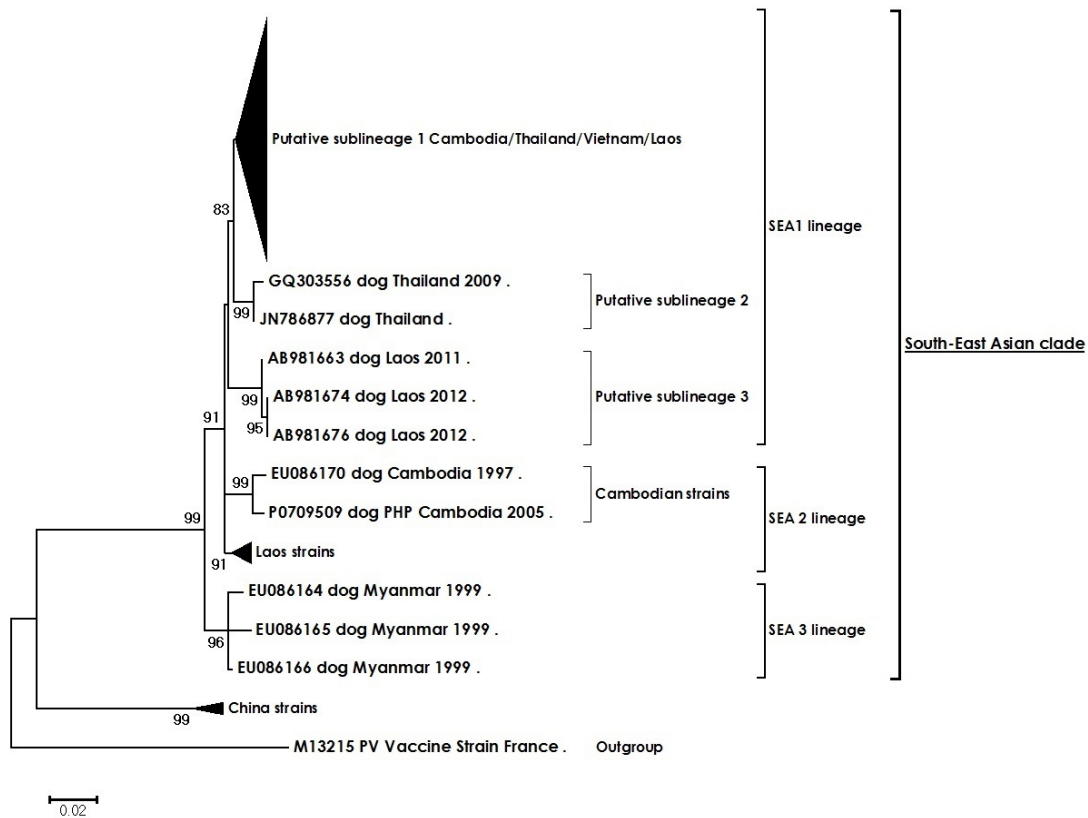


Figure 3. A. Maximum clade credibility tree of 190 complete coding region of nucleoprotein gene sequences of rabies viruses. The estimated TMRCA are indicated next to each node. Branches are color-coded by country of origin: dark blue for Cambodia, pink for Vietnam, red for Thailand, green for Laos, sky-blue for Myanmar and purple for China. The horizontal axis is shown as the number of years from the most recent sample. **B. Population dynamics of rabies viruses (complete coding region of nucleoprotein gene sequences).** The Bayesian skyline plot shows changes in relative genetic diversity (X axis) and time (Y axis) is shown as the number of years from the most recent sample. The black solid line represents the mean estimate of N_{eff} , while the 95% HPD intervals are shown in grey solid line. RABV sequence JN786877 was excluded from this analysis (absence of information regarding the date of isolation).

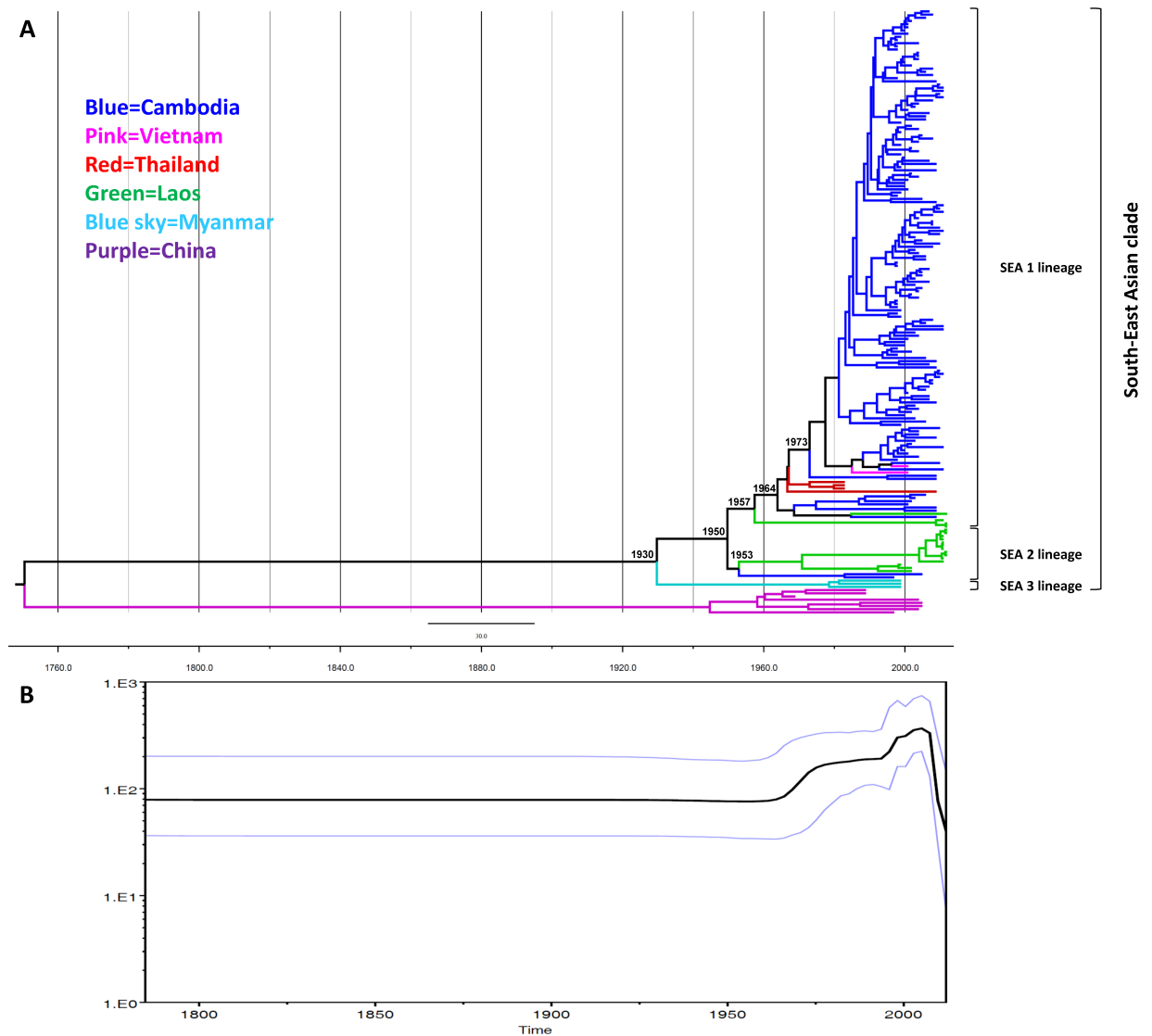


Table 1. PCR and sequencing primers used in this study

No	Name of PCR primers	Sequence 5' - 3'	References
PCR primers			
1	PV 05m	ATG ACA GAC AAY YTG AAC AA	Dacheux L. et al., 2008
2	PV 09	TGA CCA TTC CAR CAR GTN G	
3	PV 08	GGT CTG ATC TRT CWG ARY AAT A	
4	β -Taq1	TCACCCACACTGTGCCCATCTACGA	
5	β -Taq2	CAGCGGAACCGCTCATTGCCAATGG	
Sequencing primers			
1.	RVN-71F	ATGGATGCCGACAAGATTGTATTC	Yang et al., 2011
2.	RVN-1118R	GAATTCCTCTCCCAGATAGCC	
3.	RVN-1091F	CTAGGGGGCTATCTGGGAGA	
4.	RVN-1562R	CGGCCAGACCGGCTCTAACAC	

APPENDIX

Supplementary Table 1.

Information on dog rabies samples from Cambodia

№	Animal ID	Year	Age (month)	Date of sampling	Province*	GenBank accession number
1	I0119310	1998	6	19-01-98	PHP	KM366330
2	I0209267	1998	12	07-02-98	KCHA	KM366285
3	I0515204	1998	30	15-05-98	KAN	KM366251
4	I0715237	1998	24	15-07-98	KAN	KM366307
5	I0828199	1998	12	28-08-98	KAN	KM366329
6	I0918204	1998	12	18-09-98	PHP	KM366252
7	I1020180	1998	12	20-10-98	KAN	KM366332
8	I1026327	1998	36	26-10-98	KSP	KM366306
9	I1203073	1998	120	03-12-98	KCHN	KM366213
10	I1209219	1998	12	09-12-98	KCHA	KM366287
11	J0104103	1999	18	04-01-99	KAN	KM366234
12	J0122226	1999	18	22-01-99	KMP	KM366269
13	J0126058	1999	12	25-01-99	KCHA	KM366309
14	J0127108	1999	36	27-01-99	PVG	KM366241
15	J0129141	1999	10	29-01-99	PHP	KM366328
16	J0609146	1999	24	09-06-99	TAK	KM366327
17	J0701083	1999	18	01-07-99	PHP	KM366239
18	J0708136	1999	24	08-07-99	KSP	KM366295
19	J0806177	1999	60	06-08-99	KMP	KM366334
20	K0208220	2000	4	08-02-00	TAK	KM366224
21	K0329084	2000	18	29-03-00	KCHA	KM366326
22	K0424232	2000	24	24-04-00	KSP	KM366245
23	K0608309	2000	7	08-06-00	KSP	KM366210
24	K0609224	2000	7	09-06-00	PVG	KM366275
25	K0802312	2000	7	02-08-00	PHP	KM366265
26	K0816099	2000	24	16-08-00	KAN	KM366335
27	K0830060	2000	24	30-08-00	KCHA	KM366282
28	K1222058	2000	5	22-12-00	TAK	KM366224
29	L0116088	2001	3	16-01-01	KMP	KM366325
30	L0324078	2001	5	24-03-01	PHP	KM366226
31	L0411161	2001	36	11-04-01	PVG	KM366264
32	L0524176	2001	7	24-05-01	KSP	KM366278
33	L0705247	2001	3	05-07-01	KAN	KM366304
34	L0920218	2001	24	20-09-01	KTH	KM366242
35	L0925517	2001	24	25-09-01	OMC	KM366271
36	L1025187	2001	48	25-10-01	TAK	KM366321
37	L1213232	2001	24	13-12-01	KCHN	KM366240
38	L1226153	2001	5	26-12-01	KCHA	KM366290
39	M0218288	2002	36	18-02-02	KRA	KM366256
40	M0316063	2002	72	16-03-02	BMC	KM366298

41	M0506147	2002	36	06-05-02	KMP	KM366260
42	M0516129	2002	6	16-05-02	OMC	KM366209
43	M0805218	2002	12	05-08-02	PVG	KM366305
44	M0830377	2002	36	30-08-02	KAN	KM366233
45	M0906103	2002	24	06-09-02	KSP	KM366300
46	M1002140	2002	18	02-10-02	TAK	KM366215
47	M1102092	2002	12	04-11-02	KTH	KM366247
48	M1213229	2002	96	13-12-02	PHP	KM366227
49	N0120098	2003	24	20-01-03	TAK	KM366317
50	N0212228	2003	12	12-02-03	KCHA	KM366232
51	N0728149	2003	48	28-07-03	KSP	KM366218
52	N0516416	2003	12	16-05-03	PVG	KM366308
53	N0616208	2003	36	16-06-03	KAN	KM366250
54	N0711309	2003	12	11-07-03	PHP	KM366214
55	N0915299	2003	24	15-09-03	KTH	KM366324
56	N1011101	2003	24	11-10-03	KMP	KM366270
57	N1222113	2003	12	22-12-03	BMC	KM366291
58	N1229390	2003	3	29-12-03	KCHN	KM366272
59	O0109375	2004	24	09-01-04	SRP	KM366204
60	O0128528	2004	24	28-01-04	SVR	KM366302
61	O0403503	2004	36	02-04-04	PHP	KM366339
62	O0422539	2004	2	22-04-04	PHP	KM366340
63	O0527523	2004	36	27-05-04	PHP	KM366341
64	O0727536	2004	48	27-07-04	PHP	KM366342
65	O0906554	2004	36	06-09-04	PHP	KM366343
66	O1104507	2004	36	04-11-04	PHP	KM366344
67	O1106516	2004	5	06-11-04	PHP	KM366345
68	O1216508	2004	36	16-12-04	PHP	KM366349
69	O0730514	2004	24	30-07-04	PHP	KM366350
70	O0217545	2004	12	17-02-04	KCHA	KM366313
71	O0405536	2004	24	05-04-04	KCHN	KM366206
72	O0518537	2004	9	18-05-04	PVG	KM366208
73	O0625524	2004	5	25-06-04	KAN	KM366297
74	O0716535	2004	5	16-07-04	KSP	KM366219
75	O0831550	2004	12	31-08-04	KMP	KM366273
76	O1025556	2004	2	25-10-04	PHP	KM366259
77	P0324529	2005	7	24-03-05	PHP	KM366346
78	P0622512	2005	12	22-06-05	PHP	KM366347
79	P0407547	2005	3	07-04-05	SRP	KM366249
80	P0416516	2005	3	16-04-05	KCHN	KM366316
81	P0709509	2005	2	09-07-05	PHP	KM366268
82	P1110521	2005	96	10-11-05	KRA	KM366261
83	P1114533	2005	4	14-11-05	KTH	KM366312
84	Q0105521	2006	24	05-01-06	KTH	KM366292
85	Q1023563	2006	9	23-10-06	PHP	KM366348
86	Q0201551	2006	3	01-02-06	BBG	KM366203
87	Q0320591	2006	5	20-03-06	KCHA	KM366258
88	Q0420515	2006	36	20-04-06	TAK	KM366315
89	Q0428502	2006	12	27-04-06	KAN	KM366248

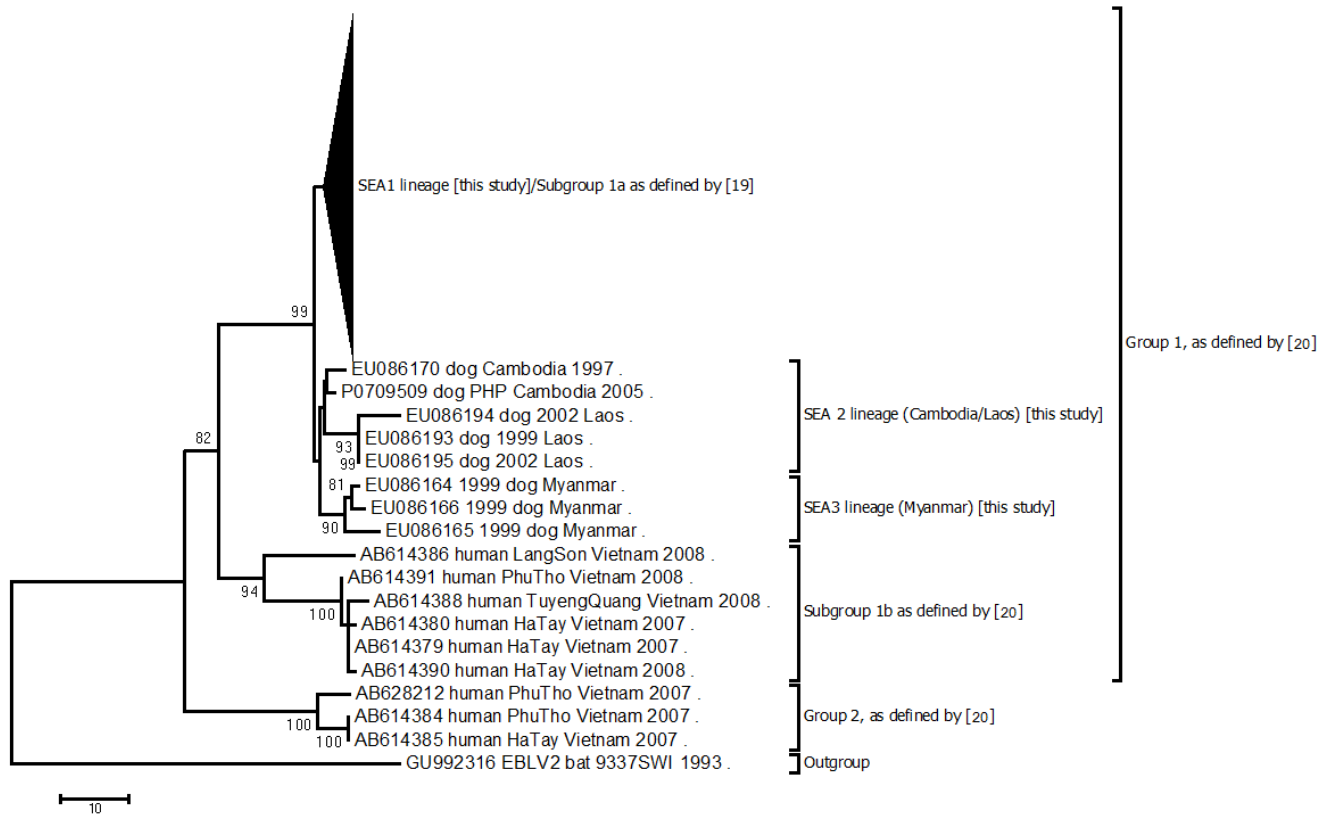
90	Q0810538	2006	36	10-08-06	PVG	KM366257
91	Q0914536	2006	12	14-09-06	KMP	KM366276
92	Q1028515	2006	3	28-10-06	KSP	KM366323
93	Q1129522	2006	3	29-11-06	KKG	KM366246
94	Q1225533	2006	24	25-12-06	PHP	KM366294
95	R0110558	2007	60	10-01-07	KSP	KM366296
96	R0222564	2007	24	22-02-07	BMC	KM366293
97	R0326539	2007	12	26-03-07	TAK	KM366243
98	R0418567	2007	2	18-04-07	KMP	KM366311
99	R0528536	2007	5	28-05-07	PVG	KM366253
100	R0802504	2007	12	02-08-07	PHP	KM366289
101	R0919544	2007	36	19-09-07	KAN	KM366207
102	R1105511	2007	24	05-11-07	KTH	KM366320
103	R1211946	2007	60	11-12-07	SVR	KM366303
104	S0407603	2008	12	07-04-08	PHP	KM366228
105	S0529625	2008	24	29-05-08	KCHN	KM366310
106	S0614621	2008	12	14-06-08	KAN	KM366212
107	S0717637	2008	72	17-07-08	KAN	KM366236
108	S0717641	2008	24	17-07-08	KAN	KM366281
109	S0804664	2008	18	04-08-08	PVG	KM366274
110	S0821644	2008	48	21-08-08	KSP	KM366231
111	S1014618	2008	24	14-10-08	SVR	KM366319
112	S1030654	2008	36	30-10-08	TAK	KM366202
113	S1103646	2008	24	03-11-08	KKG	KM366237
114	S1201660	2008	84	01-12-08	PUR	KM366280
115	S1222692	2008	24	22-12-08	TAK	KM366279
116	T0126697	2009	2	26-01-09	PRV	KM366299
117	T0216672	2009	72	16-02-09	KTH	KM366288
118	T0223715	2009	3	23-02-09	KCHA	KM366238
119	T0325655	2009	36	25-03-09	KKG	KM366217
120	T0509634	2009	24	09-05-09	KSP	KM366337
121	T0602630	2009	24	02-06-09	TAK	KM366235
122	T0617644	2009	72	17-06-09	KSO	KM366331
123	T0622656	2009	6	22-06-09	BBG	KM366283
124	T0716615	2009	48	16-07-09	PHP	KM366254
125	T0819633	2009	24	19-08-09	KCHN	KM366301
126	T0905602	2009	24	05-09-09	BMC	KM366314
127	T1002618	2009	12	02-10-09	KAN	KM366262
128	T1221671	2009	48	21-12-09	KMP	KM366333
129	U0116621	2010	12	16-01-10	KAN	KM366338
130	U0224649	2010	6	24-02-10	KCHA	KM366223
131	U0309717	2010	3	09-03-10	KCHN	KM366211
132	U0419635	2010	24	19-04-10	KMP	KM366336
133	U0520629	2010	12	20-05-10	KSP	KM366216
134	U0628690	2010	120	28-06-10	KTH	KM366318
135	U0717622	2010	12	17-07-10	PVG	KM366205
136	U0726692	2010	36	26-07-10	SVR	KM366227
137	U0821622	2010	24	21-08-10	PHP	KM366267
138	U1230640	2010	7	30-12-10	RAT	KM366266

139	V0208630	2011	5	08-02-11	BMC	KM366230
140	V0408641	2011	24	08-04-11	KAN	KM366322
141	V0411671	2011	36	11-04-11	KCHA	KM366220
142	V0520617	2011	3	20-05-11	PHP	KM366229
143	V0627625	2011	36	27-06-11	KTH	KM366222
144	V0726646	2011	12	26-07-11	KMP	KM366284
145	V0808656	2011	36	08-08-11	KRA	KM366221
146	V0926624	2011	24	26-09-11	KAN	KM366255
147	V1018638	2011	24	18-10-11	PVG	KM366225
148	V1114631	2011	72	14-11-11	KSP	KM366286
149	V1223621	2011	12	23-12-11	TAK	KM366263

*Province codes: BBG – Battambang; BMC – Banteay Meanchey; KAN – Kandal; KCHA - Kampong Cham; KCHN - Kampong Chnang; KKG - Koh Kong; KMP –Kampot; KRA – Kratie; KSO - Kampong Som; KSP - Kampong Speu; KTH - Kampong Thom; OMC –Oudor Meanchey; PHP - Phnom Penh; PRV – Preah Vihear; PUR – Pursat; PVG -Prey Veng; RAT – Ratanakiri; SRP - Siem Reap; SVR – Svay Reang.

APPENDIX

Supplementary Figure 1. Evolutionary relationships of rabies viruses from South-East Asia based on partial N-gene sequences. The tree was built using Minimum Evolution method (Rzhetsky & Nei, 1992). The optimal tree with the sum of branch length = 304.71612549 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Boot strap values lower 70 are hidden. There were a total of 381 positions in the final dataset.



Supplementary Table 2.

Information on the reference sequences

No	Strain Name	Country	Year	Species	GenBank accession #	Reference	
1.	9916CBG	Cambodia	1999	dog	EU086171	Bourhy et. al., 2008	
2.	02006CBG		1998		EU086172		
3.	9912CBG		1998		EU086169		
4.	9911CBG		1998		EU086168		
5.	9908CBG		1998		EU086167		
6.	9914CBG		1997		EU086170		
7.	01017VNM	Vietnam	2001	dog	EU086210		
8.	01016VNM		2001		EU086209		
9.	VN3		n.a.*	human	AB116579	Minoura and Nishizono direct submission	
10.	VN52		n.a.		AB116580		
11.	D 150		2006	human	AB614373	Nguyen et al., 2011	
12.	H130108		2008		AB614386		
13.	H210608		2008		AB614391		
14.	H200608		2008		AB614390		
15.	H010607		2007		AB614379		
16.	H150308		2008		AB614388		
17.	H060907		2007		AB628212		
18.	H111007		2007		AB614385		
19.	H091007		2007	AB614384	Nishizono Direct Submission		
20.	HCM2		n.a.	AB299033			
21.	HCM1		n.a.	AB299032			
22.	HCM7		n.a.	AB299036			
23.	8764THA		Thailand	1983	dog	GU992307	Vandekerkhove,J. et al., unpublished
24.	UA341			2009	human	GQ303555	Shantavasinkul,P., et al., 2010
25.	D9/52			2009	dog	GQ303556	
26.	QS-05			n.a.		JN786877	Virojanapirom et al., 2012
27.	8743THA			1983		EU086207	
28.	8734THA			1983		EU086206	
29.	9915BIR	Myanmar	1999	dog	EU086166		
30.	9909BIR		1999		EU086164		
31.	9913BIR		1999		EU086165		
32.	05008CHI	China	2004	dog	EU086189	Bourhy et. al., 2008	
33.	05009CHI		2005		EU086190		
34.	02045CHI		1989		EU086181		
35.	02044CHI		1989		EU086180		
36.	02042CHI		1969		EU086178		
37.	05007CHI		2004		EU086188		
38.	05005CHI		2005		EU086186		
39.	02037CHI		1997		EU086175		
40.	02002LAO		Laos		2002		dog
41.	9910LAO	1999		EU086193			
42.	02001LAO	2002		EU086194			
43.	Lao1	2011		AB981665	Ahmed et al., 2015		
44.	Lao2	2011		AB981663			
45.	Lao3	2011	AB981666				

46.	Lao4		2011		AB981664	
47.	Lao5		2011		AB981667	
48.	Lao7		2011		AB981668	
49.	Lao8		2011		AB981669	
50.	Lao10		2011		AB981670	
51.	Lao11		2012		AB981671	
52.	Lao12		2012		AB981672	
53.	Lao13		2012		AB981673	
54.	Lao17		2012		AB981674	
55.	Lao21		2012		AB981675	
56.	Lao22		2012		AB981676	
57.	Lao23		2012		AB981677	
58.	9337SWI	Switzerland	1993	bat	GU992316	Vandekerkhove et al., direct submission
59.	PV	France, Vaccine strain			M13215	Tordo et al., 1986