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Phylogeographic analysis of rabies viruses in the Philippines

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ABSTRACT

Rabies still remains a public health threat in the Philippines. A significant number of human rabies cases, about 200–300 cases annually, have been reported, and the country needs an effective strategy for rabies control. To develop an effective control strategy, it is important to understand the transmission patterns of the rabies viruses. We conducted phylogenetic analyses by considering the temporal and spatial evolution of rabies viruses to reveal the transmission dynamics in the Philippines.

After evaluating the molecular clock and phylogeographic analysis, we estimated that the Philippine strains were introduced from China around the beginning of 20th century. Upon this introduction, the rabies viruses evolved within the Philippines to form three major clades, and there was no indication of introduction of other rabies viruses from any other country. However, within the Philippines, island-to-island migrations were observed. Since then, the rabies viruses have diffused and only evolved within each island group.

The evolutionary pattern of these viruses was strongly shaped by geographical boundaries. The association index statistics demonstrated a strong spatial structure within the island group, indicating that the seas were a significant geographical barrier for viral dispersal. Strong spatial structure was also observed even at a regional level, and most of the viral migrations (79.7% of the total median number) in Luzon were observed between neighboring regions.

Rabies viruses were genetically clustered at a regional level, and this strong spatial structure suggests a geographical clustering of transmission chains and the potential effectiveness of rabies control that targets geographical clustering. Dog vaccination campaigns have been conducted independently by local governments in the Philippines, but it could be more effective to implement a coordinated vaccination campaign among neighboring areas to eliminate geographically-clustered rabies transmission chains.

1. Introduction

Rabies is a fatal viral disease caused by the rabies virus (RABV), which is a negative-sense RNA virus belonging to the family *Rhabdoviridae*. Although effective control measures including dog vaccination are available, rabies still causes an estimated 55,000 human deaths annually worldwide, primarily in Asia and Africa (Knobel et al., 2005). In the Philippines, rabies remains a public health threat and approximately 200–300 human rabies cases are reported annually according to the National Notifiable Diseases Surveillance System (Department of Health and the Philippines, 2009). The annual incidence rate of human rabies is approximately 0.2–0.3/100,000, which is still higher than neighboring countries including Vietnam, Malaysia, and Indonesia (Global Infectious Diseases and Epidemiology Network Informatics, 2010). The National Rabies Control and Prevention Program of the Philippines, a joint effort by the Department of Agriculture, Department of Health, and other partners, set a target of 2020 for the elimination of rabies from the country (Department of Health and the Philippines, 2009;

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Dodet, 2010). Therefore, an effective strategy for rabies control is urgently required for the country.

The rabies epidemiology and the concept of "How rabies spreads" needs to be investigated for the development of a feasible rabies control program (Lembo, 2012; Partners for Rabies Prevention, 2010), and phylogenetic analysis is one of the useful methods to understand the transmission dynamics in the country. Many statistical methods have recently been developed, and the phylodynamics has undergone some of the most advances in recent years (Holmes, 2008; Pybus and Rambaut, 2009). A phylogeny can be constructed by considering trait evolution such as temporal and geographical traits; both molecular clock and phylogeographic analyses have been developed to observe the temporal and spatial transmission dynamics of infectious diseases (Faria et al., 2012; Lemey et al., 2009b; Nunes et al., 2012; Talbi et al., 2010).

According to previous studies, the Philippine strains were introduced from China and formed a unique phylogenetic cluster called Asian 2b (Bourhy et al., 2008; Gong et al., 2010; Meng et al., 2011). Our previous study indicated that there are three major clades in each island group, i.e., Luzon, Visayas, and Mindanao and several sub-clades in different geographical locations, suggesting an independent evolution in each area without frequent introduction into other areas (Saito et al., 2013). However, only the descriptive study was performed, and the transmission dynamics of RABVs in the Philippines is yet to be defined. To reveal the transmission dynamics of RABVs, we conducted phylogenetic and phylogeographic analyses that considered temporal and spatial evolution. We aimed to define the evolutionary history and diffusion process of RABVs in the Philippines, which will aid in establishing more effective rabies control strategies.

2. Material and methods

2.1. RABVs data set

To define the transmission dynamics of RABVs in the Philippines, 233 RABV complete glycoprotein (G) gene sequences (1572 nucleotides) were analyzed using phylogenetic inferences. Details of the methods including the sample collection and sequence analysis were previously reported (Saito et al., 2013). Briefly, RABV G sequences were obtained from brain tissue samples of suspected rabid animals (mainly dogs) from 11 of the 17 regions in the Philippines between 2004 and 2010 (Table S1). The Philippines consists of three island groups (Luzon, Visayas, and Mindanao) and is administratively divided into regions, provinces, municipalities/cities, and barangays. The 11 regions included Regions I, II, III, IV-A, IV-B, V, the Cordillera Administrative Region (CAR), and the National Capital Region (NCR) in Luzon; Region VII in Visayas; and Regions X and XI in Mindanao (Table S1). All reference sequences used for the comparative analyses were obtained from GenBank.

This study protocol was approved by the Institutional Review Board of the Research Institute for Tropical Medicine (RITM).

2.2. Phylogenetic analyses

The multiple sequence alignment was performed using ClustalW implemented in MEGA 5.0 (Tamura et al., 2011), followed by recombination screening using RDP v3.44 (Martin et al., 2010). In the analyses, a general time reversible model with gamma distribution (GTR+4Γ) was selected as a substitution model of the G gene using the Akaike Information Criterion with a correction value in the model selection procedure implemented in MEGA 5.0 (Tamura et al., 2011).

The molecular clock analysis was performed based on the Bayesian Markov Chain Monte Carlo (MCMC) analysis using continuous-time Markov chains that were implemented in the BEAST package (Drummond et al., 2012). In this analysis, 30 reference sequences of Asian 2 strains including the Asian 2a (n = 19, Chinese strains obtained from GenBank) and the Asian 2c (n = 11, Southeast Asian strains obtained from GenBank) were added into the data set of the Philippine strains (n = 233), so that the diverging history of the Philippine strains (Asian 2b) from the other Asian 2 strains could be analyzed. The sampling year was used as temporal data, and the evolutionary history was estimated after considering the relaxed uncorrelated log-normal clock model as well as a strict clock model. A Bayesian skyline model or a constant population size model was used as a tree prior (Drummond et al., 2006, 2005). A posterior set of trees (PSTs) was obtained through Bayesian MCMC analysis, which was run for 300,000,000 iterations for each model and sampled every 30,000 states to obtain an effective sample size (ESS) (greater than 200). The ESS was calculated by Tracer program v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The marginal likelihood analysis was performed to calculate the Bayes factors (Suchard et al., 2001) for each combination of clock and tree prior model with 1000 bootstrap replicates using the Tracer program; an additional two MCMC analyses were run using the best-supported combination under Bayes factors. After removing 10% of the burn-in, three runs were combined using the LogCombiner program implemented in the BEAST package. The Maximum clade credibility (MCC) tree was constructed from the PSTs using the TreeAnnotator program in the BEAST package and visualized with the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/).

The reversible discrete phylogeographic analysis was performed using the BEAST package (Lemey et al., 2009a; Drummond et al., 2012) along with the BEAGLE library (Suchard and Rambaut, 2009) to estimate the diffusion process of the RABVs in the Philippines. To complete the estimation of the diffusion process in the Philippines, only the Philippine strains were included in this analysis (n = 233). The largest administrative boundary, "regions," was used as a discrete location state, and the sampling date was used to calibrate the time scale. As with the molecular clock analysis, the preliminary MCMC analyses were performed for each combination of clock and tree prior model, and three MCMC analyses were run using the best-supported combination under Bayes factors. After removing 10% of the burn-in, those runs were combined to construct an MCC tree. The MCC tree was converted to a keyhole markup language file using SPREAD (Bielejec et al., 2011) and projected into a map using ArcGIS 10 (ESRI Inc, Redlands, CA, USA). Shape files for maps of the Philippines were obtained from the Global Administrative Areas website (http://www.gadm.org/country). To identify significant migrations, the Bayes factor testing was performed using the Bayesian stochastic search variable selection (BSSVS) procedure (Lemey et al., 2009a). The migrations with Bayes factors that were greater than five were summarized as well-supported migrations (Lemey et al., 2009b). To count the number of migrations between locations, the Markov jump counts (Minin and Suchard, 2008; Talbi et al., 2010) along the branches of the PSTs were estimated (O'Brien et al., 2009).

To analyze the spatial structure of viral transmission, the association index (AI), indicating the strength of phylogenetic clustering by traits (Wang et al., 2001), was calculated from the PSTs after the first 10% of tree states were removed as burn-in using BaTS software (Parker et al., 2008). The PSTs were constructed using the data set from the Philippine strains. The index ratio of the observed mean AI values to the expected mean AI values (null set) was calculated to indicate the strength of the association between the traits and the phylogeny. In the phylogeny of whole Philippine strains, sampling year and sampling location (regions

and island groups) were coded for traits to observe the strength of temporal and spatial structure of RABV transmissions in the Philippines. The null set was calculated by 100 replicates of the state randomization of phylogeny-trait association.

To exclude the possibility that genetically identical samples could affect the results of time calibration and trait estimation, another data set ($n = 194$) was constructed by excluding any genetically, spatially, and temporally identical samples from the full-data set (Table S1). Samples collected from the same region and in same year were excluded. This data set was used for the additional MCMC runs for the molecular clock analysis, the Bayes factor testing, and the AI calculations with/without 30 reference strains. The output was compared with the results obtained using the full-data set (including 233 Philippine strains with/without 30 reference strains).

2.3. Amino acid sequence evolution

The G protein amino acid sequences of the Philippine strains were analyzed to reveal the mutations in the antigenic and glycosylation sites, which are associated with viral antigenicity, pathogenicity, and transmissibility (Benmansour et al., 1991; Coulon et al., 1998; Dietzschold et al., 1983; Flamand et al., 1993; Lafon et al., 1984, 1983; Marissen et al., 2005; Seif et al., 1985; Wunner et al., 1985; Yamada et al., 2012). The glycosylation sites were predicted using NetNGlyc 1.0 Server (Gupta et al., 2004). To define the amino acid evolution from other Asian 2 strains, the amino acid sequences of other Asian 2 strains were also included in this analysis.

Selective pressure on the G gene of the Philippine strains was estimated using the single likelihood ancestor counting (SLAC) method and the fixed-effects likelihood (FEL) method using the Datamonkey webserver (Delport et al., 2010; Kosakovsky Pond and Frost, 2005; Pond and Frost, 2005). We also used a mixed-effects model of evolution (MEME), which allows selective pressure to vary from site to site and branch to branch at a site, to detect site-by-site episodic positive selection (Murrell et al., 2012). The relative rates of nonsynonymous (dN) and synonymous (dS) substitutions were calculated and then the mean ratio (dN/dS) and site-by-site (and branch to branch for MEME) selection were estimated to detect any selective pressure on the RABVs through evolutionary history in the Philippines. The positive or negative selections with p-values of <0.1 were considered statistically significant in the SLAC and FEL methods, and p-values of <0.05 were considered significant in the MEME method because the SLAC/FEL methods are more conservative and result in fewer false positives (Kosakovsky Pond and Frost, 2005; Streicker et al., 2012).

3. Results

3.1. Evolutionary history of RABVs

There were no recombination signals detected among the 263 sequences including the 233 Philippine strains and 30 reference Asian 2 strains. Thus, all sequences were included in the following analyses. Based on the marginal likelihood analysis, the highest Bayes factor was observed in the combination of the uncorrelated log-normal relaxed clock and the Bayesian skyline model, although there were no significant differences for the estimates among other combinations of 2 clock and 2 tree prior models (Table 1 and S2). Using the best-supported combination, three Bayesian MCMC analyses were run, and they converged to almost same estimates in each parameter. Those runs were combined, and a MCC tree was constructed (Table 1 and Fig. 1). The coefficient of variation of this combined relaxed clock was less than 1 [0.418 with the 95% highest posterior density (HPD) interval of 0.244–0.598], and this coalescent-based analysis was suitable for further analyses (Drummond et al., 2006). The phylogenetic relationships and evolutionary history of the Asian 2 strains, including the Philippine strains, are shown in Fig. 1. All of the Philippine strains belonged to the same cluster, Asian 2b; the Asian 2b strains had diverged from the Chinese strains (Asian 2a), as has been shown in previous studies (Gong et al., 2010; Meng et al., 2011) (Fig. 1). Through the molecular clock analysis, the nucleotide substitution rate and time to the most common ancestor (tMRCA) were estimated. The substitution rate of the Asian 2 cluster was estimated to be 5.81×10^{-4} substitutions/site/year (95% HPD: $4.47–7.27 \times 10^{-4}$); the date that the MRCA of the Asian 2 cluster existed was estimated to be 1831 (95% HPD: 1764–1894). After 1896 (95% HPD: 1850–1938), the Asian 2a and 2b cluster diverged. The date that the MRCA of Asian 2b existed was estimated to be 1967 (95% HPD: 1954–1981); since then, the Asian 2b cluster has diverged into 3 major clades (Luzon, Visayas, and Mindanao clades) with high posterior probability. All of the Philippine strains, except for 2 strains from Luzon, were included in these 3 major clades, according to their geographical boundaries, i.e., island groups. Those 2 strains were collected from Region IV-A, located in the southern part of Luzon, and Region IV-B, an island region located south of Luzon. The posterior probability values for the common ancestors of these 2 strains were considerably low; both of them were less than 50%, showing their distinctness from the three major clades.

These results were not affected by the genetically identical samples because no significant difference was observed in the estimates of the nucleotide substitution rate and the MRCA between the two data sets, i.e., the full data set ($n = 263$) and another data

Table 1
Estimates of nucleotide substitution rate and the date that the most recent common ancestor (MRCA) existed in different clock and tree prior models.

	Strict clock		Uncorrelated log-normal relaxed clock		Uncorrelated log-normal relaxed clock Bayesian skyline (3 MCMC runs combined)[b]
	Constant size	Bayesian skyline	Constant size	Bayesian skyline	
Nucleotide substitution rate (/site/year)	5.01 (4.03–6.04) × 10^{-4}	4.99 (3.92–6.00) × 10^{-4}	5.86 (4.37–7.26) × 10^{-4}	5.82 (4.48–7.14) × 10^{-4}	5.81 (4.47–7.27) × 10^{-4}
Date MRCA existed					
Asian 2 cluster	1814 (1769–1854)	1813 (1767–1854)	1831 (1762–1895)	1833 (1766–1894)	1831 (1764–1894)
Asian 2a and 2b cluster	1880 (1848–1909)	1879 (1848–1909)	1895 (1846–1936)	1896 (1850–1938)	1896 (1850–1938)
Asian 2b cluster	1960 (1948–1972)	1959 (1946–1971)	1969 (1955–1982)	1967 (1954–1981)	1967 (1954–1981)
ln$P$ (model|data)[a]	−10993.00 (S.E. ± 0.54)	−10995.01 (S.E. ± 0.53)	−10963.45 (S.E. ± 0.62)	−10963.29 (S.E. ± 0.68)	–

The results of the first Markov Chain Monte Carlo (MCMC) run for marginal likelihood analysis are shown for each combination of the models.
The mean [and the 95% highest posterior density (HPD) intervals] is indicated for the substitution rate and the date MRCA existed.
[a] 1000 replicates of bootstrap had been performed for calculating marginal likelihood.
[b] Three MCMC runs had been performed and combined using the best supported combination of the clock and tree prior model under the Bayes factors (uncorrelated log-normal relaxed clock and Bayesian Skyline model).

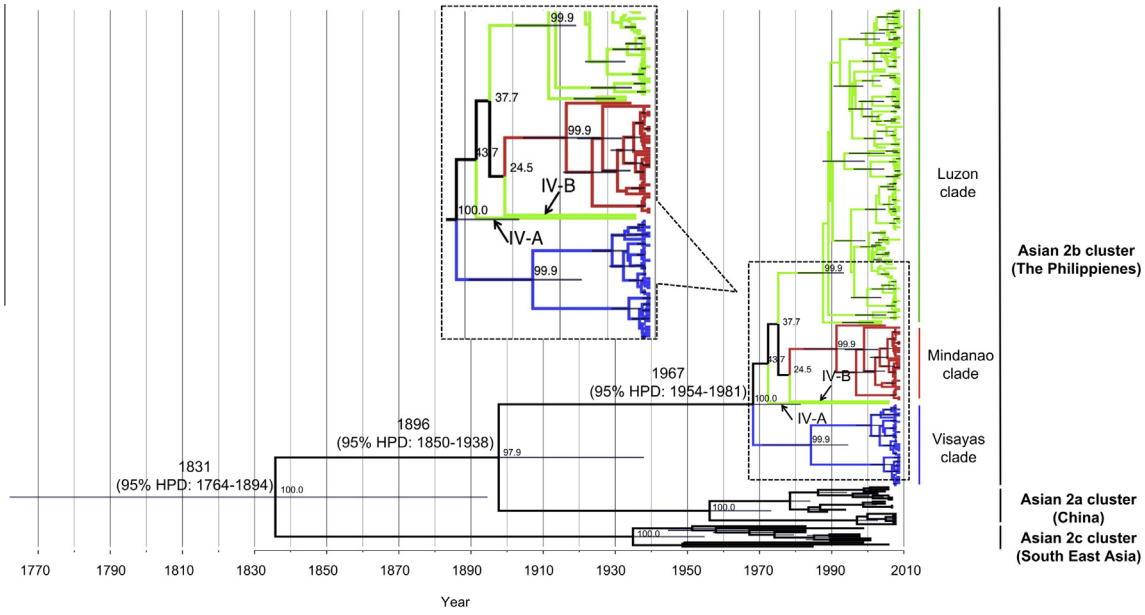

Fig. 1. Maximum clade credibility tree of Asian 2 strains, including the Philippine strains (Asian 2b). The phylogenetic relationships and temporal evolutionary history have been estimated by molecular clock analysis. Branch lengths are temporally scaled, and the *x*-axis shows the time unit (year). Asian 2a (Chinese strains), Asian 2b (Philippine strains), and Asian 2c (South East Asian strains) have been included in this analysis, and Philippine strains are colored according to the island groups located (Green, Luzon; Red, Mindanao; Blue, Visayas). Two distinct Philippine strains are indicated by arrows with the name of each region where they were collected. The most recent common ancestors (MRCAs) of Asian 2, Asian 2a and 2b, and Asian 2b are indicated, and the dates that these MRCAs existed are also shown on the nodes. The 95% highest posterior density (HPD) interval is shown on the node with >70% posterior probability. The posterior probability values are indicated only for major nodes.

set excluding genetically, spatially, and temporally identical samples ($n = 224$) (Table S3).

3.2. Diffusion process of RABVs in the Philippines

The marginal likelihood for the phylogeographic analysis was summarized in Table S4. Similar to the molecular clock analysis, the combination of the uncorrelated log-normal relaxed clock and Bayesian skyline model was best supported under the Bayes factor (Table S4). Using these models, the reversible discrete phylogeographic analysis was performed to analyze the diffusion process of the Philippine strains at a regional level (Fig. 2). In the Philippines, the RABVs diffused from Region IV-A to other island groups beyond the sea (Fig. 2A and B). After these island-to-island migration events, the RABVs only migrated within each island group, i.e., from Region X to Region XI in Mindanao, from Region IV-A to other regions in Luzon. Since then, no more island-to-island migration was observed. The significant migrations, which were supported by Bayes factors of greater than five, were summarized in Table 2. All of the significant events were migrations within the same islands, and no significant island-to-island migrations were observed. The Bayes factor testing using another data set ($n = 194$) showed no difference between the two data sets ($n = 194$ and $n = 233$) (Table S5).

When we focused on the diffusion process in Luzon, the RABVs mostly migrated from one region to neighboring regions that share a regional land border (Fig. 2A). The Markov jump counting also showed that 79.7% of migrations, calculated using median values, were observed between neighboring regions, and it was significantly higher than that between nonneighboring regions in Luzon ($p < 0.0001$; One-sided Wilcoxon rank sum test) (Fig. 3). However, some long-distance migrations between nonneighboring regions were also observed particularly from/to Region V (Fig. 2A). In addition, there was one significant long-distance migration event between Region V and NCR, which were more than 100 km away from each other (Table 2).

3.3. Spatial structure of RABV transmission in the Philippines

The AI statistics were calculated to estimate the strength of the temporal and spatial association with the RABV transmission pattern in the Philippines. The island group and region data were assigned in the PSTs as spatial traits, and the sampling year was assigned as a temporal trait. The results indicated a much stronger association with spatial structure rather than with temporal structure in RABV transmissions in the Philippines (Table 3). Among the spatial traits, the index ratio for an island group was relatively lower than that for a region, although strong spatial structure was still observed at a regional level. These results were not affected by genetically, spatially, and temporally identical samples, as shown in Table S6.

3.4. Amino acid substitutions among the Philippine strains

Amino acid substitutions in the antigenic sites were summarized in Table 4. Among the Philippine strains, only a few mutations in the antigenic sites were detected. Among all Asian 2 strains included in the analysis, one amino acid mutation in the antigenic site VI (R264H) was detected in the Asian 2a strains compared with Asian 2b and 2c strains; however, most of the Asian 2 strains had the same sequences in the antigenic sites. All the Philippine strains and most of other Asian 2 strains had the same sequons at position 37 (N-L-S) and 319 (N-K-T) of the G gene, a commonly-detected glycosylation site in RABV (Wunner et al., 1985; Yamada et al., 2012). One Asian 2a1 strain (GuizhouAl03/Guizhou/2005) and one Asian 2c strain (YunNanTc06/Yunnan/2006), did not possess the sequon at position 37 (N-L-P). Two Philippine strains (TRa-279 and TRa-283, detected in Region II) had one more glycosylation site at position 247, which was a previously reported glycosylation site (Wojczyk et al., 2005; Wunner et al., 1985; Yamada et al., 2012).

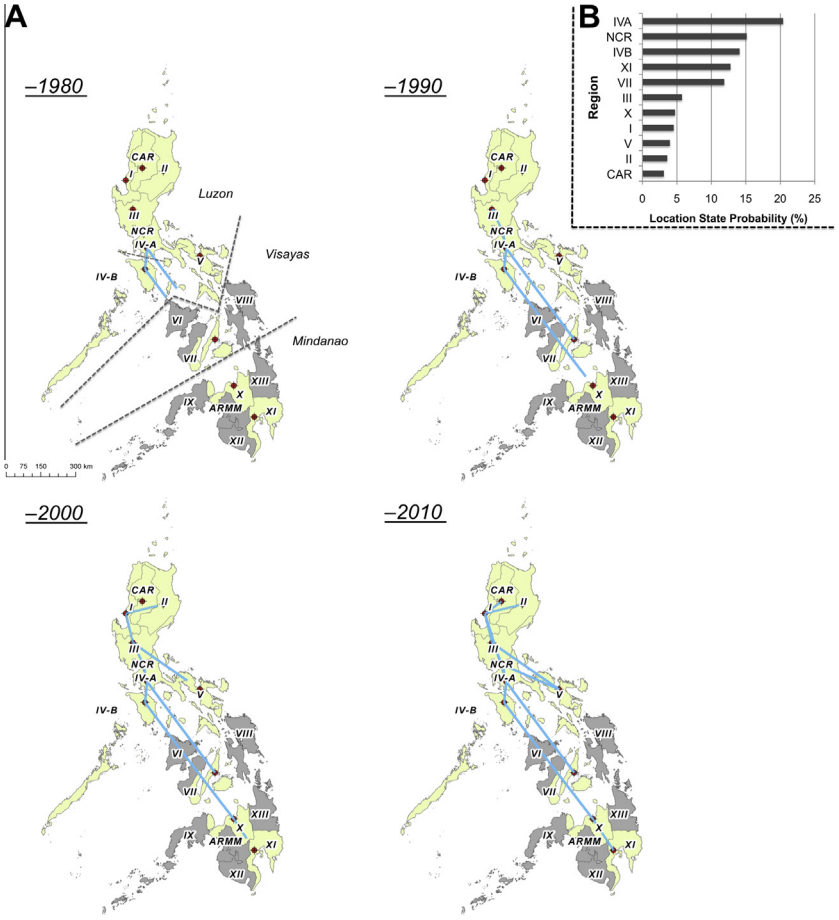

Fig. 2. Diffusion process of RABVs in the Philippines. The regional diffusion process has been estimated using a Maximum Clade Credibility tree of the Philippine strains by reversible discrete phylogeographic analysis. (A) The diffusion process is divided into four time periods, and each process is projected into the Philippine map by straight blue lines. The names of each island group and region are indicated on the map, and data-missing regions are masked by gray color. (B) Location state probability of the most recent common ancestor (MRCA) of the Philippine strains is indicated in the graph. Region IV-A is estimated to be the most probable location of MRCA in the Philippines. CAR, The Cordillera Administrative Region; NCR, The National Capital Region.

Table 2
The migrations well-supported by Bayes factors.

Region		Bayes factor[a]
IV-A	NCR	5883.7
CAR	I	2538.4
I	III	1125.9
III	NCR	82.4
NCR	V	30.1
X	XI	9.9

The migrations supported by Bayes factor greater than five are indicated. CAR, The Cordillera Administrative Region; NCR, The National Capital Region.

[a] Bayes factor testing had been performed by the Bayesian stochastic search variable selection procedure.

3.5. Selective pressure through evolutionary process in the Philippines

Finally, to observe whether the Philippine strains experienced any selective pressure through their evolutionary history, the ratio of *dN* and *dS* was calculated. Therefore, the mean *dN*/*dS* value was 0.121 with a 95% confidence interval range of 0.103 and 0.141 in SLAC. No positively selected site with statistical significance ($p < 0.1$) was detected, except for one site where there was evidence of episodic diversifying selection at position 154 in the ectodomain of G gene, as detected by MEME ($p = 0.0049$).

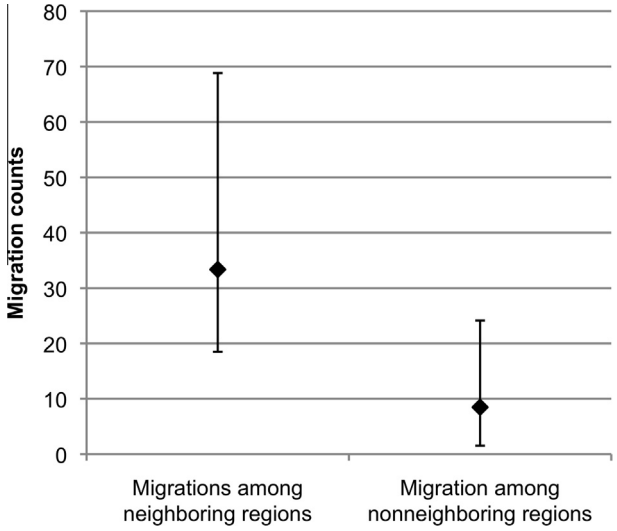

Fig. 3. The number of migration events in Luzon. The number of migration events among neighboring regions and nonneighboring regions in Luzon had been estimated by counting Markov jumps along the branches of posterior set of trees. The median and 95% highest posterior density intervals are indicated in the graph.

Table 3
Association index (AI) values for spatial or temporal data.

Traits	Index ratio[a] (95% CI[b])	AI value (95% HPD CIs[b])		*p*-Value
		Observed mean	Null mean[c]	
Year	0.523 (0.418–0.646)	8.79 (7.60–9.93)	16.8 (15.4–18.2)	<0.01
Region	6.62 (4.66–8.64) $\times 10^{-2}$	1.51 (1.11–1.88)	22.8 (21.8–23.8)	<0.01
Island group	6.57 (0.00–18.0) $\times 10^{-15}$	8.61 (0.00–21.4) $\times 10^{-13}$	13.1 (11.9–14.3)	<0.01

The AI value has been calculated using posterior set of trees of Philippine strains.
[a] Index ratio: observed mean/null mean.
[b] HPD CIs: highest posterior density confidence intervals.
[c] The null distribution was yielded by 100 trait randomization.

Table 4
Amino acid mutations in the antigenic sites.

Antigenic sites	Mutations	Minor strains	Clusters
Anitigenic site I (226–231)	K226R	TRa-220/Region I/2009	2b: Philippine strain
		TRa-259/Region I/2009	2b: Philippine strain
	V230I	TRa-065/Region III/2008	2b: Philippine strain
	L231P	JX08-47/Jiangxi/2008	2a2
Antigenic sites II (34–42, 198–200)	S39P	YunNanTc06/Yunnan/2006	2c
		GuizhouAl03/Guizhou/2005	2a1
	F41L	Z-06-203/Region IV-B/2006	2b: Philippine strain
Antigenic site III (330–338)	V332I	Z-04-480/Region IV-A/2004	2b: Philippine strain
	N336D	Z-06-203/Region IV-B/2006	2b: Philippine strain
Antigenic site VI (264)	R264H	TRa-191/Region X/2009	2b: Philippine strain
		TRa-248/Region X/2009	2b: Philippine strain
		TRa-256/Region I/2009	2b: Philippine strain
		TRa-258/Region I/2009	2b: Philippine strain
		Asian 2a strains	2a

4. Discussion

In the Philippines, rabies remains a significant public health issue and approximately 200–300 human rabies cases are annually reported. Since dog bites are the major cause of human rabies infections in the country (Armbulo et al., 1972; Department of Health and the Philippines, 2009; Dodet, 2010), a canine rabies eradication program, e.g., mass dog vaccination campaign, is possibly the most effective strategy for rabies control (Denduangboripant et al., 2005; Nagarajan et al., 2009). Understanding how RABVs are transmitted in the community could be essential to establishing a more effective dog vaccination strategy (Lembo, 2012; Partners for Rabies Prevention, 2010). Therefore, we tried to analyze the evolutionary history and diffusion process of RABVs using recently developed statistical frameworks to reveal the transmission dynamics of RABVs in the Philippines.

From this study, the Philippine strains, belonging to the Asian 2b cluster, were shown to have diverged and been introduced from China around the beginning of the 20th century (Fig. 1). Following this introduction, no further introduction from other countries was observed. The RABVs in the Philippines evolved to form three major clades according to the geographical boundaries of the island groups of Luzon, Visayas, and Mindanao. Within the Philippines, RABVs have diffused from Luzon to Visayas and Mindanao, across the sea (Fig. 2A). Migrations between island groups were observed only once during 50 years, and since then, RABVs have spread and evolved only within each island group, i.e., from Region X to Region XI in Mindanao and from Region IV-A to other regions in Luzon. Although the most probable origin of the Philippine strains appeared to be Region IV-A, the probability was not significantly high compared with other regions (Fig. 2B); we could not detect any significant island-to-island migration after the Bayes factor testing with the BSSVS procedure (Table 2). These results may have been affected by the two distinct strains detected in Region IV-A and IV-B in Luzon. Both of them had ancestors with low posterior probability, indicating uncertainty in the estimation of the origin and destination of the island-to-island migrations.

In the estimated evolutionary history, a long temporal gap period was observed between the date of origin introduction and the major divergence in the Philippines (Fig. 1). Since the first recorded rabies case was documented in 1918 (Department of Health and the Philippines, 1918, 1951), there may be other missing processes in the evolutionary history. The two distinct strains were not included in any clades, which may indicate that these two strains were part of the missing processes. These distinct strains were not detected in other regions of Luzon, although the Luzon clade RABVs were circulating on the entire island. This suggests the following two possibilities: these two strains could not migrate to other regions in Luzon or these two strains continued to circulate only in these regions although similar strains had already been replaced by the Luzon-clade. In the former possibility, the reason why these strains could not migrate to other regions is not known, given that the MRCA of Luzon clade could diffuse to the entire area of Luzon. The ancestors of these strains may not have been able to maintain their transmission chains due to virological factors, e.g., antigenicity, pathogenicity, or transmissibility, or they may not have been able to diffuse or transmit due to epidemiological factors, e.g., geographical barriers. It was not known if there were any differences in the antigenicity or pathogenicity between the ancestral and descendant viruses because we did not perform virological characterization of these strains. However, virological factors may not be the reason since only sporadic amino acid mutations and no episodic diversifying selection were detected in the antigenic sites in the amino acid sequence. In the latter possibility, the reason why the ancestors of these strains disappeared in other parts of Luzon may be explained by a genetic bottleneck

(Holmes et al., 2002; Wittke et al., 2002). Because the reproduction number (R_0) of rabies is not high, (R_0 = 1–2; Coleman and Dye, 1996; Hampson et al., 2009), some strains may disappear in the stochastic process of their evolutionary history, and we may not have detected the turnover of these strains during our relatively short study period due to the generation time. The missing evolutionary processes in Visayas and Mindanao can be explained by this theory. In Visayas and Mindanao, missing processes may have existed in the evolutionary history, since the annual report of the Philippines health services reported rabies cases in Luzon and Visayas in 1918, and in Mindanao in 1951 (Department of Health and the Philippines, 1918, 1951). However, the Visayas and Mindanao clades were genetically too close to the Luzon clade to determine if the ancestors existed before 1918 or 1951. There could have been strains with other genetic properties, which existed in those island groups that stochastically disappeared and were replaced by ancestors of the Visayas and Mindanao clades in their evolutionary history. It is also necessary to consider the sampling bias because there are still some areas where no RABVs have been collected because of the passive sample collection method used in the study. We do not know whether the descendants of these missing evolutionary processes disappeared or were simply not detected in this study. To observe those evolutionary processes, further sampling from regions should be continued for the analysis of the phylogenetic relationship with other major three clades.

Although we experienced uncertainty in our estimation of evolutionary processes, we showed that the transmission pattern was strongly shaped by geographic boundaries. Since the AI statistic showed a lower index ratio for the Island group than other traits, the Philippine strains had strong spatial structure within each island group, indicating the sea as a significant geographical barrier for viral transmission (Table 3). Even at a regional level, strong spatial rather than temporal structures were still observed. In the diffusion process, most of the migrations were observed between neighboring regions (Figs. 2A and 3), and these results also indicate the strong spatial structure in RABV transmissions. RABVs were genetically and spatially clustered at a regional level, and virus migrations primarily occurred from neighboring regions through their land borders. This strong spatial structure indicates geographical clustering of transmission chains and the effectiveness of rabies control programs targeting this geographical clustering. The dog vaccination campaign has been conducted by each local government in the Philippines, and it could be more effective to implement vaccination campaign in coordination with neighboring local governments to eliminate geographically-clustered transmission chains.

However, some long-distance migrations were also observed, particularly from/to Region V, and some of RABVs migrated to non-neighboring regions. Because domestic dogs are the primary reservoirs for rabies in the Philippines and generally stay close to the human community (Beran, 1982; Childs et al., 1998; Pal et al., 1998; Rubin and Beck, 1982), such long-distance migrations may not have occurred only through dog movements. The study of Talbi et al. (2010) reported human-mediated dispersal of RABVs. Although we could not detect any evidence for human-mediated dispersal of RABVs in this study, long-distance migrations of viruses, including initial island-to-island migrations, most likely occurred through occasional human-mediated dog movement. Despite its low frequency, we need to pay attention to these long-distance migrations for rabies control.

Rabies is a multi-host zoonosis, and cross-species transmission is possible. However, as mentioned above, domestic dogs are reported to be a primary reservoir in the Philippines because this country has few wild carnivorous animals (Lawrence et al., 2010). The major wild animals are bats and rats, and it was reported that they had not been included in the transmission chains of rabies (Beran, 1982). In the Philippines, RABVs did not experience any selective pressure through their evolutionary process as estimated through the SLAC and FEL; we could detect only one episodic diversifying selection at position 154 in the G gene, which was not located on any antigenic sites or in sites that may be associated with viral fitness and/or pathogenicity (Benmansour et al., 1991; Coulon et al., 1998; Dietzschold et al., 1983; Flamand et al., 1993; Holmes et al., 2002; Lafon et al., 1984, 1983; Marissen et al., 2005; Seif et al., 1985). Since the host shifts during cross-species transmission can involve several positive selections including an antigenic site (Streicker et al., 2012), our results may support the report that RABVs transmissions have been sustained in dog communities and have not been circulated into any other population in the Philippines (Armbulo et al., 1972; Beran, 1982).

There are several limitations in this study. The study period was relatively short, and the evolutionary history may not be completely reconstructed. There may be other missing processes in the evolutionary history, and we could not detect those in this study. In addition, we analyzed the strains based on wider geographical administrations, i.e., 11/17 regions of the Philippines. However, there remain many geographic areas where no RABVs have been analyzed. Moreover the number of samples analyzed per region also varied and may have been temporally as well as spatially biased because sample collections were totally dependent on regional laboratory capacity and voluntary submission from inhabitants (Saito et al., 2013). Despite these limitations, we believe our analyses provide valuable information on viral evolution and transmission dynamics of RABVs in the Philippines.

5. Conclusions

In the Philippines, there is a national goal of eliminating rabies by 2020 (Department of Health and the Philippines, 2009; Dodet, 2010), and for this purpose, rabies control programs have been conducted by each local government. However the program has not been systematically implemented with the cooperation of neighboring areas. An effective strategy for rabies control is urgently required in the Philippines. In this study, we focused on the evolutionary history and diffusion process of RABVs in the country using Bayesian inference methods. Migrations between island groups were observed only once in this 50 year period, and the transmission patterns were strongly shaped by geographical boundaries. RABVs were genetically and spatially clustered at the regional level, and most of the migrations were observed between neighboring regions. This strong spatial structure indicates the geographical clustering of transmission chains and the effectiveness of rabies control programs that target these geographical clusters.

As a next step, a more detailed local transmission pattern can be informative for rabies control strategies for the local administrative authorities. The factors (e.g., social factors, ecological factors, or landscape features) that may be associated with the strong spatial structure in the transmission dynamics in the Philippines need to be further investigated. For this objective, the local transmission pattern of RABVs should be revealed using a geographic information system and/or ecological approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.01.026.

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