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Evolutionary dynamics of rabies viruses highlights the importance of China rabies transmission in Asia

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ABSTRACT

Rabies in Asia is emerging as a serious public health issue. To explore the possible origin, phylogenetic relationships, and evolutionary dynamics of Asian Rabies viruses (RABV), we examined 200 complete nucleoprotein (N) gene sequences from RABV isolates in the region. Phylogeny supported the classification of Asian RABVs into five distinct clusters in lyssavirus genotype 1. Our geospatial and temporal analyses demonstrated that China appears to be the prime source of Asian RABVs. Understanding of rabies transmission and associated human activities, such as dog translocation, can help rabies control and elimination in Asia through collaborative efforts or programs.

Introduction

Rabies in Asia is widely distributed, and has been well documented in regional ancient civilizations such as China and India (Yu, 2001). Although rabies is endemic in the region, how the disease is transmitted from country to country in Asia is largely unclear. Currently, India and China have the most human rabies cases in the world (WHO, 2005). This situation could be a reflection of the natural history of rabies in endemic countries. Rabies in Asia has never been controlled except for a few island countries or regions. Domestic dogs play a primary role in rabies transmission, and are responsible for more than 95% of human rabies cases in China and India (Nagarajan et al., 2009; Si et al., 2008). Since rabies transmission is affected by domestic dog population density (Zinsstag et al., 2009), and rapid turnover of domestic dog populations has been a major obstacle to successful rabies control in developing countries (Hampson et al., 2009), the origin of rabies should be closely related to the origin of domestic dogs. Strong evidence supports that dogs were domesticated from gray wolves in Southeast Asia (very possibly in China) less than 16,300 years ago (Pang et al., 2009). China and India subcontinent could be the ideal regions to study the origin and transmission dynamics of canine rabies. Dog rabies viruses (RABVs) comprise six major clades: Africa 2, Africa 3, Cosmopolitan, Arctic-related, Asian and Indian subcontinent clades. The last three clades circulate extensively in Asia, with the Arctic-related clade circulating in a large region extending from central Asia (former Soviet Union) to eastern Asia (Russia, Nepal, Pakistan, northeastern Iran, the north of India and Korea)(Nadin-Davis et al., 2007). The Asian clade is widely distributed in Southeast Asia, including Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia, Philippines and China (Bourhy et al., 2008). The Indian subcontinent clade is distributed only in southern India and Sri Lanka (Bourhy et al., 2008; Drummond et al., 2005).

Although RABVs are responsible for classical rabies in Asia, their origin, transmission patterns and divergence remain unknown. Large-scale phylogenetic analysis of RABVs sequence data can reconstruct the epidemiological history of viral populations (Pybus and Rambaut, 2009) and lead to better understanding of RABV transmission and improve rabies control programs.

In this study, we investigated the transmission history of RABVs in Asia by conducting a comprehensive evolutionary analysis of spatial and temporal spread of 200 RABVs sampled from East and Southeast Asia over a time period of 78 years. We inferred that Time to the Most Recent Common Ancestor (TMRCA) of Asian RABVs ranges from 1526

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to 1794 AD (95% highest posterior density (HPD)) and China appears to be the primary source of Asian RABVs and rabies transmission in the region.

Results

Phylogenetic relationships

The maximum-parsimony (MP) analysis presented five distinct clusters of Asian RABVs: Asian 1, Asian 2, Asian 3, Arctic-related and Cosmopolitan clusters (Fig. 1 and Fig. S1) with strong bootstrap (BT) values (BT = 100). Our Bayesian Markov Chain Monte Carlo (BMCMC) phylogenetic analysis also revealed 5 clusters of RABV circulating in Asia (Fig. 2). Each cluster was defined by long branches and nodes supported by high Bayesian posterior probability values (P>90%). The spatial structure could even be treated as a single cluster. The Asian cluster 1 has been isolated from at least 15 provinces and 3 municipalities (Beijing, Chongqing and Shanghai) in China and has one isolate from Indonesia. This cluster seems to be the most widespread in China, which is compatible with previous studies using G gene analyses (Meng et al., 2007; Zhang et al., 2009). The Asian cluster 2 is broadly distributed in China and Southeast Asia, namely Myanmar, Thailand, Laos, Cambodia and Philippines. The Asian cluster 3 was derived from isolates in Sri Lanka and India and was called Indian subcontinent clade. The Arctic-related cluster circulated in Nepal, Afghanistan, South Korea, India and China (Nadin-Davis et al., 2007). Finally, the Cosmopolitan cluster included isolates from Mongolia and China. The geographical locations of the different genetic clusters of Asian RABVs are shown in Fig. 3. Our study is the first comprehensive genetic analysis of Asian RABVs recovered from 13 counties in Asia, and confirms extensive circulation of the Asian 1, Asian 2 and the Arctic- related clusters in Asia.

Timescale of RABV evolution

We employed a BMCMC approach to estimate the timescale of emergence of Asian RABVs. The mean rate of nt substitution for the N gene in the dataset was estimated to be 4.316×10^{-4} substitutions per site year^{-1} (95% HPD, 3.11×10^{-4}-5.632×10^{-4} substitutions per site year^{-1}), which was concordant with previous estimates (Bourhy et al., 2008; Talbi et al., 2009). The TMRCA of Asian RABVs by BMCMC analysis ranged from 1526 to 1794 AD (95% HPD), which was consistent with the report by Gong et al. (2010). A similar substitution rate and TMRCA were observed by simple root-to-tip regression distances from the Neighbor-Joining (NJ) tree that was generated under the general time reversible (GTR) evolutionary model of evolution (Fig. 4), confirming sufficient temporal structure in our Asian RABVs dataset to support this estimation.

Population and spatial dynamics of Asian RABVs

To trace the time and location in RABVs emergence and transmission in Asia, we plotted the geographical location of each cluster onto the map (Fig. 3). The Monte Carlo analysis generated migration events and frequency distributions for each entry in the resulting migration matrix (Table S1-4) by delayed transformation (DELTRAN) and accelerated transformation (ACCTRAN) optimization. A sparse false discovery rate (sFDR) correction applied to the multiple comparisons test identified 6 pathways with migration significantly greater than expected under the Monte Carlo distributions under ACCTRAN optimization: Cambodia to China, Cambodia to Laos, Cambodia to Thailand, Nepal to India, Nepal to Afghanistan, and Sri Lanka to India (Table 1). The 15 and 9 migration events accumulated considerable support even as their P values missed their sFDR cutoff

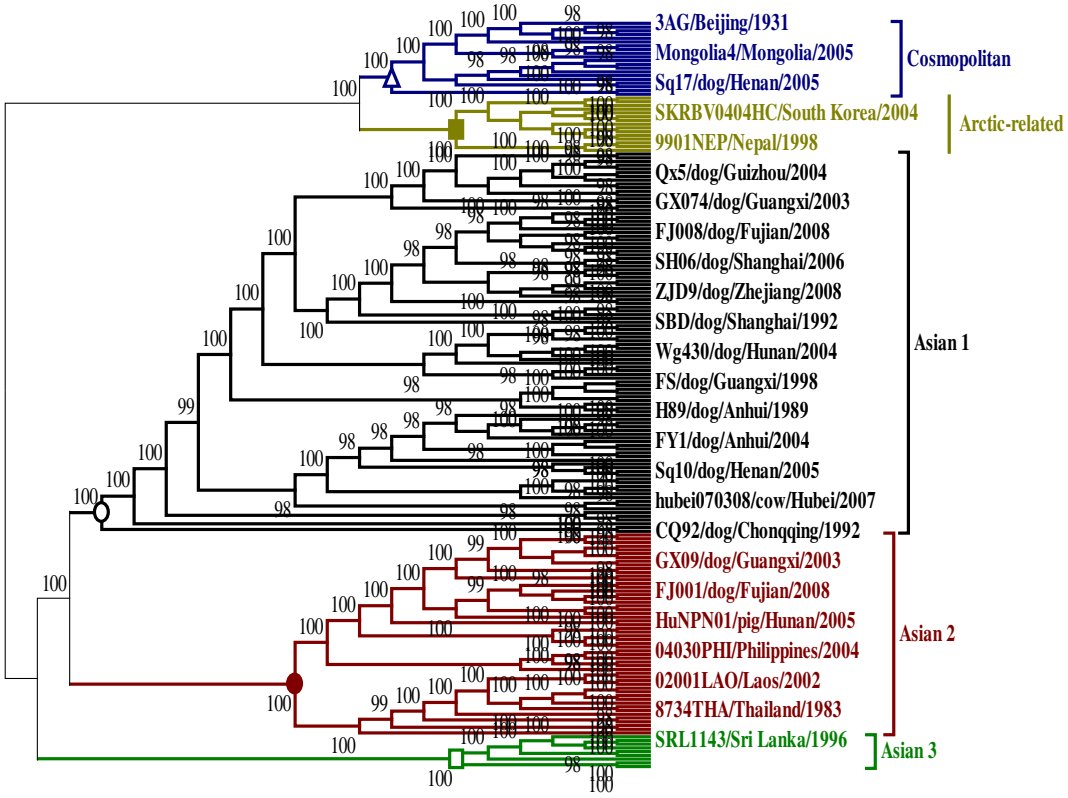

Fig. 1. Phylogenetic tree of 200 Asian RABVs N gene sequences using the maximum-parsimony analysis (MEGA 4). Percentage bootstrap values above 70% are shown at the branch nodes.

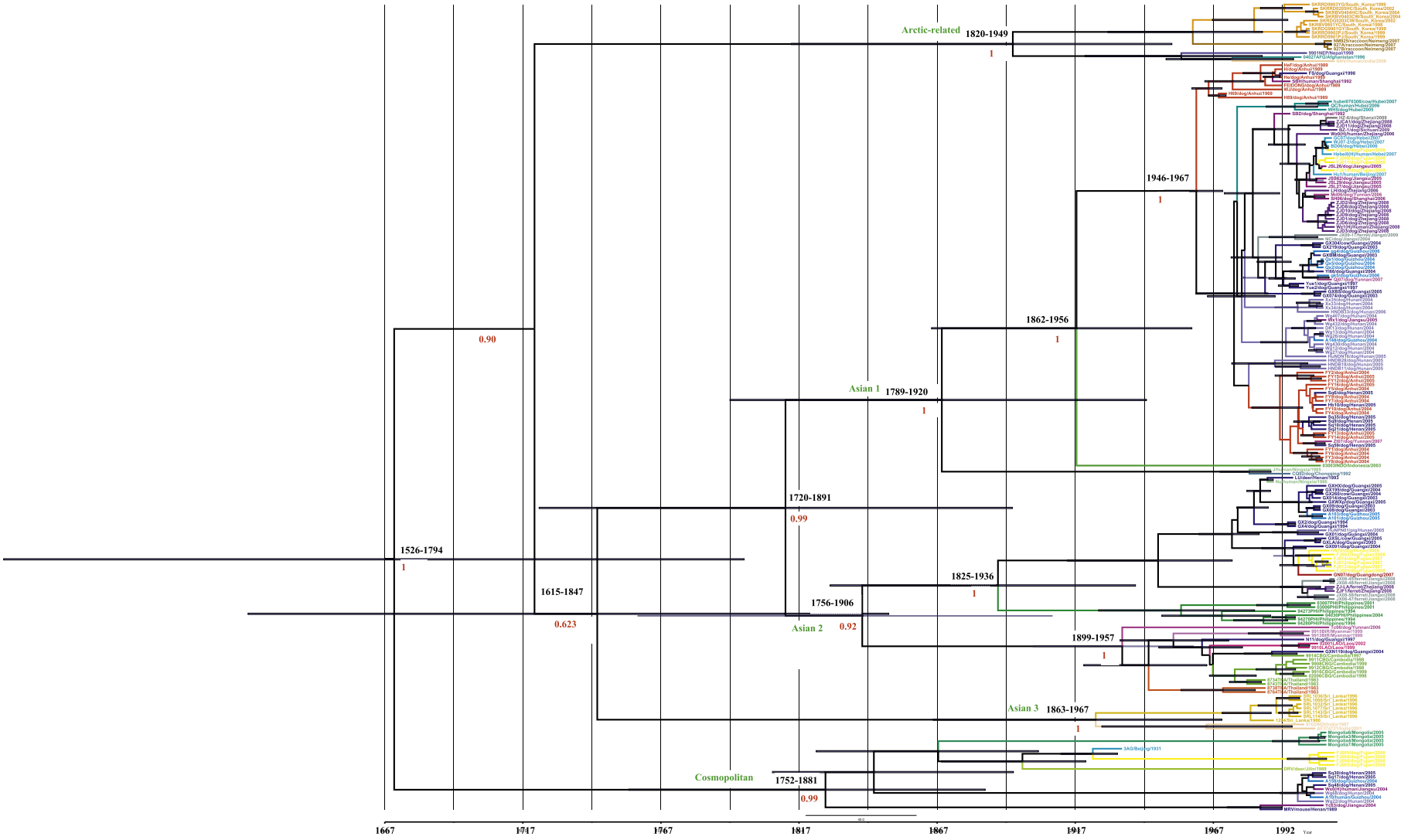

Fig. 2. MCC tree of 200 Asian RABVs N gene sequences. The horizontal branches are drawn to a scale of estimated year of divergence. Upper and lower limits of the 95% HPD estimates, and the corresponding divergence dates for the five clusters of Asian RABVs sequences are shown. Posterior probability values are shown for key nodes.

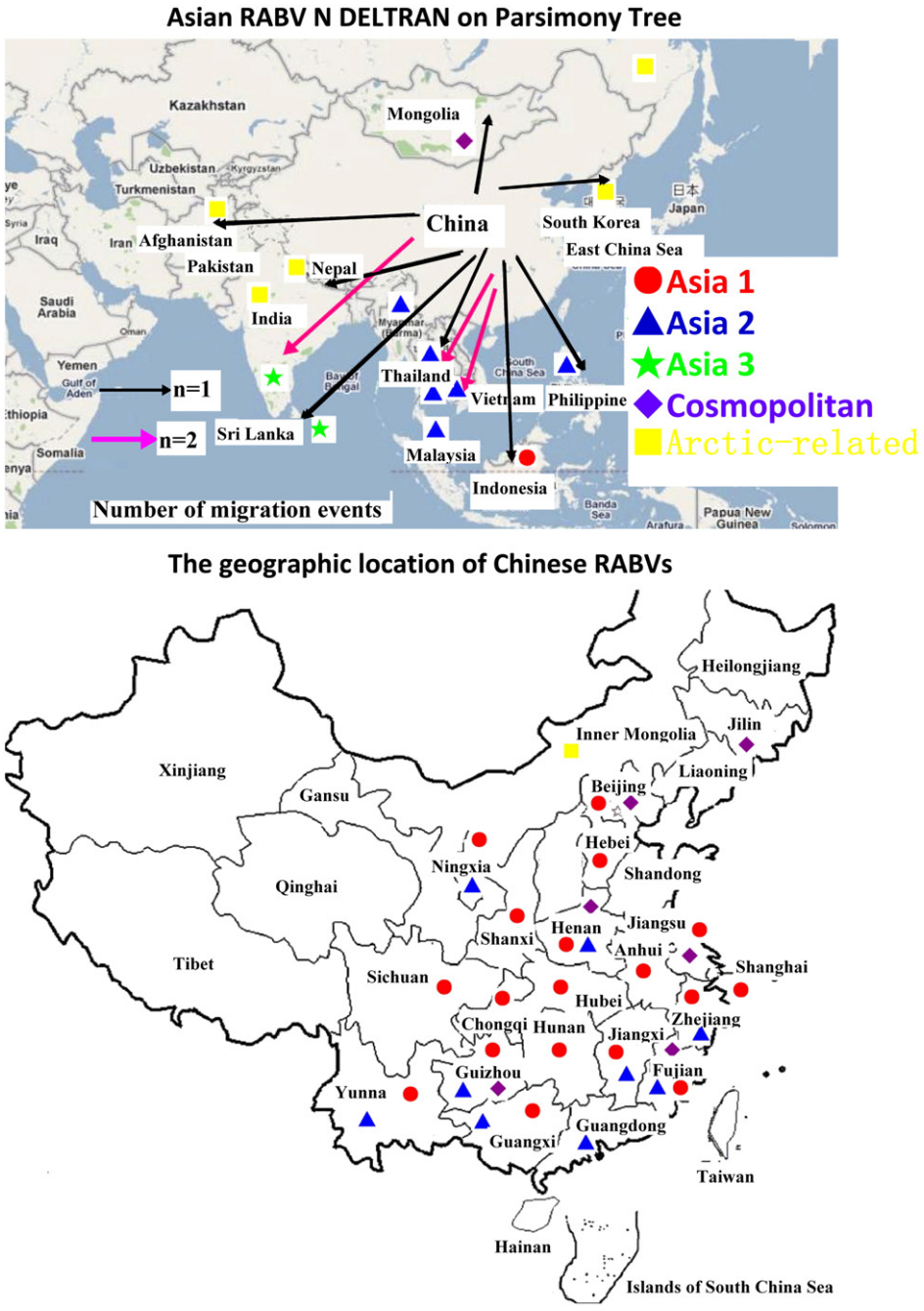

Fig. 3. Map of geographical distribution and DELTRAN RABV migration events inferred through maximum- parsimony phylogeny for 200N nucleotide sequences sampled across 13 Asian countries. Arrows indicate migration direction of Asian RABVs from China to other countries. Different clusters are indicated by colors and shapes.

under DELTRAN and ACCTRAN optimization, respectively (Table 1). After we combined these analyses with geographic information system data analyses, we found that China appears to be the prime source of Asian RABVs (Fig. 3), which was probably due to human interventions.

Discussion

Rabies is still a serious veterinary and public health problem in Asia. Molecular epidemiological studies can provide information on virus origins and spread that can facilitate development of effective rabies control programs (Metlin et al., 2007). Our phylogeographic study of 200 RABV samples from 13 countries in Asia provided some epidemic history of rabies in Asia and demonstrated that China appears to be the primary source of Asian rabies events (Fig. 3 and Table 1).

In this study, the earliest common ancestor of present RABV variants in China and Southeast Asia was estimated to be from 1526 to 1794 AD (95% HPD), in accordance with the previous report (Gong et al., 2010), in which the divergence time of RABVs analyzed by 126G gene sequences (113 from China and 13 from 8 Southeast Asian countries) was estimated to be from 1514 to 1812 AD (95% HPD), but was different when only Chinese RABVs sequences were analyzed (Meng et al., 2010; Ming et al., 2010). In our study, TMRCA of Asian 1 and Asian 2 was estimated within a relatively narrow range of 1720–1891 AD (95% HPD). The earliest common ancestor estimation is inconsistent with records of dog rabies in China, which trace the disease back to 556 BC (Wu et al., 2009). The extinct ancient RABVs

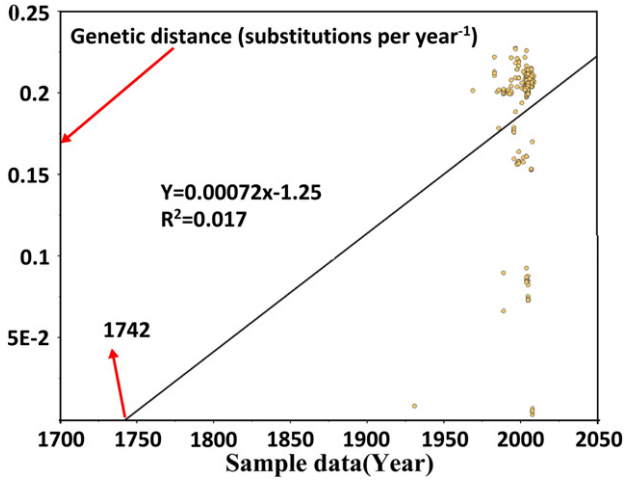

Fig. 4. Regression of root-to-tip distances against sampling date for the N gene sequences of Asian RABVs. The inferred rate of nucleotide substitution (slope), time to common ancestry (intercept), and correlation coefficient (r^2) are also shown.

that no longer circulate may have been responsible for early rabies outbreaks. Our temporal reconstruction indicates that RABVs circulating in Asia have a common ancestor likely from China. The divergence time range of Asian 1, 2 and 3 clusters could be traced back to 1789–1920, 1756–1906 and 1863–1967 AD (95% HPD), respectively, and the common ancestor of Asian 1, 2 and 3 clusters was present in 1615–1847 AD(95% HPD). These evolutionary dynamics are compatible with the timeline of human emigration from southern China to Southeast Asia in the last few centuries. Many Chinese emigrated to Southeast Asia during the late Ming and early Qing dynasties. During this time, human-related activities, such as persons migrating with infected dogs may have contributed to the long-distance spread of rabies.

Our MigraPhyla analysis indicated that RABVs from China diffused into the other 12 Asian countries. Animal trade and dog relocation associated with human activities have been implicated in rabies outbreaks across Southeast Asia (Nadin-Davis et al., 2007; Tao et al., 2009). Our previous study suggested rabies translocation events between provinces in China due to human-associated activities (Meng et al., 2010; Tao et al., 2009). Hunan province may have acted as a source of rabies to the other provinces (Meng et al., 2010). Similarly in this study, our phylogenetic and MigraPhyla analysis also indicated that RABVs circulating in Asia have a common ancestor, likely from China. We also did not find that TMRCA of RABVs from southern Indian subcontinent (Asian 3 cluster) was earlier than those mainly from China (Asian 1 and Asian 2 clusters). The majority of the migration events involved non-contiguous geographical areas with long distance migrations, which most likely reflect human intervention. More RABVs sequences from other Asian countries may reveal more transnational migration events.

Rabies continues to impact human health despite the existence of proven cost-effective control measures. Vaccinating domestic dogs against rabies results in a significant reduction in the incidence of human rabies and this control strategy has been shown to be the most cost-effective for medium to long-term goals; costs are typically recouped within 5–10 years, mainly through decreased expenditure on human post-exposure treatment (Knobel et al., 2005; Meslin et al., 1994). At the same time, detailed studies on dog ecology and rabies epidemiology and treatment in Asia are necessary to resolve this issue.

This study provides new insights into the pathways of spatial diffusion and virus migration across Asia and illustrates the epidemiological characteristics of RABVs in the region. This information will help set up risk evaluation measures to prevent rabies transmission and develop control measures that will benefit public health. We hope this report will encourage further studies to apply similar molecular approaches to understand the complexity of rabies situation in Asia and spur efforts to improve public awareness as well as increase international collaboration in rabies control.

Table 1
Parsimony analysis of migration events, *P* values and sFDR among isolates of RABV were inferred using the complete N gene sequences sampled across 13 Asian countries. The bold shows migration event (with *P* values < sFDR).

		DELTRAN			ACCTRAN		
From	To	Migration events	*P* values	sFDR	Migration events	*P* values	sFDR
a	b	1	1	0.0566	1	1	0.0666
a	c	1	1	0.1	1	1	0.11
a	d	2	1	0.1433	1	1	0.1533
a	e	1	1	0.1866			
a	f	1	1	0.23	1	1	0.2366
a	g	1	1	0.2733	1	1	0.28
a	h	1	1	0.3166	1	1	0.3233
a	i	1	1	0.36	1	1	0.3666
a	j	2	1	0.4033	1	1	0.41
a	k	2	0.9999	0.01			
a	l	1	0.9986	0.0033	1	0.9986	0.0233
a	m	1	0.9989	0.0066			
d	**a**				**1**	**0.0012**	**0.0167**
d	**e**				**1**	**0.0013**	**0.02**
d	**j**				**1**	**0.0008**	**0.0133**
h	**k**				**1**	**0.0001**	**0.0033**
h	**m**				**1**	**0.0001**	**0.0067**
i	**k**				**1**	**0.0007**	**0.01**

a:China, b: Philippines, c: South Korea, d: Cambodia, e: Laos, f: Mongolia, g: Myanmar, h: Nepal, i: Sri Lanka, j: Thailand, k: India, l: Indonesia, m: Afghanistan

Materials and methods

Viruses and RT-PCR

From 1969 to 2009, we collected 55 RABV isolates in China (Table S5). Total RNA was extracted (TRIzol reagent; Invitrogen, Carlsbad, CA, USA) from brain tissue according to the manufacturer's instructions. The complete RABV nucleoprotein (N) gene was amplified by RT-PCR using primers listed in Table 2 (Meng et al., 2009). After electrophoresis, the amplified products were purified using the Agarose Gel DNA Purification Kit (TaKaRa Biotechnology Co., Dalian, China) and were then directly sequenced on Applied Biosystems 3730 DNA Analyzer using the ABI-PRISM Dye Termination Sequencing Kit (Applied Biosystem Inc., Foster City, CA, USA).

Asian RABVs data sets

We chose full length N (1,353 nucleotides-nt) gene sequences for our analyses. The data set included 154 Chinese isolates (Table S5) and all available RABVs from other Asian countries in GenBank (Table S6), presenting the largest data set so far for Asian RABVs between 1931 and 2009. The new sequences in this study have been submitted to GenBank and assigned accession numbers were EU159362 to EU159387 and HQ118101 to HQ118118.

Table 2
Primers used in this study.

Name	Sence	Viral locus	Sequence(5′ to 3′)	Nucleotide position	Use
RV1-F	+	3'UTR	GTACCTAGACGCTTAACAAC	1–11	PCR/Sequencing
RHN-2	−	N	TCTGCTCTATCCTATCCGCAATG	584–562	PCR/Sequencing
N128	+	N	ATGTAACACCCCTACAATGG	55–74	PCR/Sequencing
N81	−	N	CAGTCTCCTCAGCCATCTC	1567–1586	PCR/Sequencing

Phylogenetic and BMCMC evolutionary analyses

To construct the phylogenetic tree, a set of 200 complete N gene sequences including 55 of the present study and 145 retrieved from GenBank, was calculated using the MP and NJ methods in the MEGA package, version 4 (Kumar et al., 2008) . The statistical significance of the reconstructed phylogenies was estimated by bootstrap analysis with 100 (MP) or 1000 (NJ) replicates. Bootstrap values above 70% were considered significant (Hillis and Bull, 1993).

The rate of nt substitution per site (substitutions per site year^{-1}) and TMRCA for the complete N gene datasets were estimated using the BEAST package, which employs a BMCMC approach, utilizing the number and temporal distribution of genetic differences among viruses sampled at different times (Drummond and Rambaut, 2007). The GTR model incorporating invariant sites was found to be the best fit for the data according to the Akaike information criterion. A previous analysis of RABV evolution found that the uncorrelated log-normal relaxed clock and constant population size model provided a better fit to the data than the uncorrelated exponential model (Bourhy et al., 2008), and this was confirmed in a preliminary analysis of a subset of the data collected here, although the parameter values estimated were similar under both models. The BMCMC analyses contained 2×10^8 states, with sampling every 1000 states, and the first 10% of each chain was discarded as burn-in. Convergences and effective sample sizes (effective sample size of all relevant parameters >200) of the estimates were checked using Tracer v1.4 (http://beast.bio.ed.ac.uk/Tracer). The Maximum Clade Credibility (MCC) tree across all of the plausible trees generated by BEAST was then computed using TreeAnnotator, version 1.4.8 (http://beast.bio.ed.ac.uk/) and Figtree, version 1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/), and samples were colored according to their location of sampling. The degree of uncertainty in each parameter estimate is provided by 95% HPD values, while posterior probability values provide an assessment of the degree of support for each node on the tree. To assess the reliability of our estimates of both the substitution rate and TMRCA, and to determine the extent of temporal structure for Asian RABVs sequence data, we performed a regression analysis of tree root-to-tip genetic distance against sampling date based on NJ trees using the program Path-O-Gen (http://tree.bio.ed.ac.uk/software/ pathogen/).

Geospatial and temporal analysis

To determine the extent and pattern of geographic structure in the RABVs, we assigned each sequence in the "total" data set a single-letter character state reflecting the location of origin. Character state changes were ordered by outgroup comparison with isolate 3aG/Beijing/1931.The migration events through the tree were traced with MigraPhyla (Wallace et al., 2007; HoDac et al., 2007). The method infers the localities of the tree nodes by maximum parsimony, and by Monte Carlo simulation test for the significance of migration events. A Monte Carlo test of 10,000 trials was conducted to determine the probability that the frequencies of migration events (P values) between each pair of localities in the original migration tree were more than expected when the localities were randomly distributed across the tree's tips. To test against the null hypothesis of completely unrestricted migration between geographical states, the mean number of observed state changes was compared with the frequency. The Monte Carlo P values of significant pathways are less than their sFDR (sparse false discovery rate) cutoff: P value rank$\times$(0.05/total migration events). The test is capped at a distribution conservative enough to account for a regular spatial distribution in which each hypothesized migration occupies a different cell in the migration matrix. The migration events, P values and sFDR between these geographical states were inferred based on the refined ML phylogeny independently using two parsimony optimization methods, called ACCTRAN (in which events are forced to occur as soon as possible moving from the root to the tips of the tree) and DELTRAN (in which events are delayed as soon as possible) implemented.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.12.011.

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