

## The Full-length Genome Analysis of a Street Rabies Virus Strain Isolated in Yunnan Province of China\*

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**Abstract:** The epidemic of rabies has rapidly increased and expanded in Yunnan province in recent years. In order to further analyze and understand the etiological reasons for the rapid expansion of rabies in Yunnan, a strain of rabies virus CYN1009D in Yunnan was isolated, and the complete genomic sequencing was carried out, and the bioinformative analysis on genes/encoded proteins and phylogeny with reference to sequences in GenBank was performed. The complete genome of CYN1009D was 11923 nt in length and belonged to genotype I. The genes encoding different structural proteins were all conserved in their lengths, in comparison to other strains in China. The amino acid sequence was conserved at different antigen sites of NP, but the variation was detected at the secondary phosphorylation site of position 375; variations were also detected in the phosphorylation sites at positions 63-63 and 162 of PP; the sites playing important roles in virus synthesis, budding and viral morphology in MP were conserved; two glycosylation sites were detected at Asn37 and Asn319 in GP, the neutralizing antigen sites in GP were conserved; the initial amino acid of LP (ML) was different from that of most of the strains in China (MM); the variations in G-L region in the intergenic region were significant. The phylogenetic tree showed that CYN1009D has a closer genetic relationship to the strains in Southeast Asia, indicating that prevention and control on rabies in borderland areas should be reinforced meanwhile efforts are made to control rabies in China.

**Key words:** Rabies virus; Yunnan CYN1009D; Complete genome; Characterization

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Rabies virus (RABV) belongs to the *Mononegavirale* order, the *Rhabdoviridae* family and the *Lyssavirus* genus. The genome is a non-segmented, negative-sense, single-strand RNA which is about 12,000 nucleotides (nt) long. This viral RNA encodes five structural proteins in order: Nucleoprotein (NP), phosphoprotein (PP), matrix protein (MP), glycoprotein (GP) and large protein (LP).

Sequencing and analysis on the complete genome of rabies virus were started in 1988<sup>[20]</sup>, and more than 70 strains of rabies viruses have been subjected to complete genome sequencing according to NCBI index till 2011. The complete genome sequencing of the rabies virus has important significance for completely understanding molecular evolution, gene regulation and genetic variation of the rabies virus.

China is a country with high morbidity for rabies and ranks the second highest place in the world after India. Rabies also leads in the number of reported deaths among the various kinds of communicable diseases of the Notifiable Communicable Disease Reporting System in China. The reported cases mainly occur in south China, and among them more than 90% of the cases are found in Yunnan, Guangdong, Guangxi, Hunan and other provinces or autonomous regions<sup>[23]</sup>. It should be noticed in these areas with high morbidity, such as in Yunnan province, the reported human rabies cases (The data was collected as part of the "National Disease Reporting Information System" of the Chinese Center for Disease Control and Prevention) has rapidly increased and expanded in recent years (Fig. 1). Only one or few cases was reported in 2000, 2002 and 2003 respectively in Yunnan, and rabies showed scattered prevalence before 2005 but it rapidly increased in 2006, 108 cases were

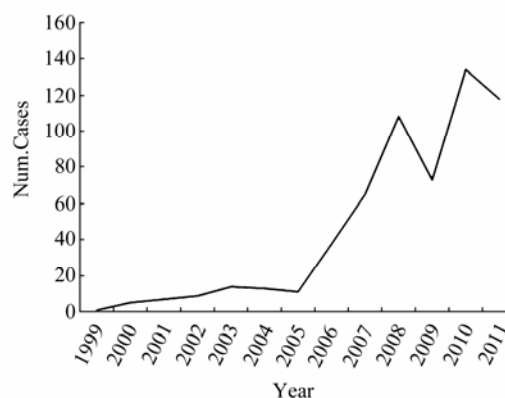


Fig. 1. Rabies cases in Yunnan province from 1996 to 2011.

reported in 2008 and 134 cases were reported in 2010.

Yunnan province with its special geographic location is adjacent to Tibet, Sichuan, Guizhou and Guangxi provinces of which Sichuan, Guizhou and Guangxi are areas with a high incidence of rabies. It is also adjacent to Laos, Burma, Vietnam, and close to Thailand where rabies is prevalent.

In recent years, the full-length genomes of some vaccine and street strains of rabies viruses were determined<sup>[3,13,16,18]</sup>, which establish the foundation for understanding the characteristics of hereditary variation for rabies virus in China. In this study, we isolated and sequenced a street strain of rabies virus at the borderline area of Yunnan, with an aim to explore the correlation between the rapid increase and expanding of rabies in Yunnan and the hereditary variation of rabies virus.

## MATERIALS AND METHODS

### Virus strain

The CYN1009D strain was isolated from the brain tissue of a rabid dog in Lianghe county, Dehong Prefecture, Yunnan province (as shown in Fig. 2), and subjected to 1 passage in the suckling mice to amplify

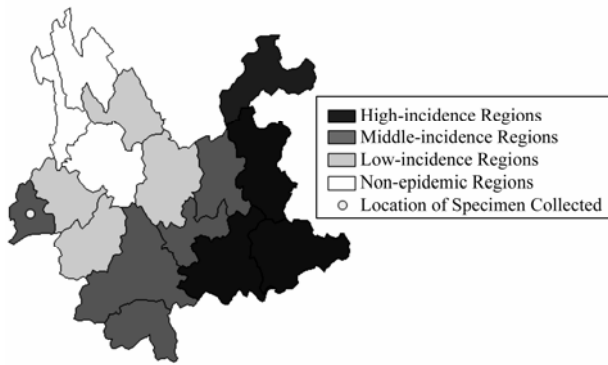


Fig. 2. Geographical distribution of rabies cases in Yunnan from 1999 to 2011.

the virus. The brain tissue of the moribund suckling mice was used to isolate the total RNA.

### Primer design

In total, 16 pairs of primers spanning the entire CYN1009D genome were designed using Primer Premier, version 5 (PREMIER Biosoft International, CA, USA), and all primers were designed within the conserved regions of the genome, determined from an alignment of all the full genomic sequences of the reference strains (Table 1).

Table 1. Primers for amplification of the whole genome of CYN1009D strain

Fragment no.	Primer <sup>a</sup>	Sequence(5'-3')	Length(bp)	Position <sup>b</sup>
1	RV1-P1	CAGGTAGACGCTTAACAAC	1032	1-1032
	RV1-P2	TGACCCATATAGCATCCAAC		
2	RV2-P1	CAGGAGACTGCTGTTCTCA	869	899-1767
	RV2-P2	TCATCCATCTGGAAGTCCTC		
3	RV3-P1	TAACACCCCTCCTTTCGAACCAT	1018	1485--2502
	RV3-P2	TACGCCCGTTATCAGTGGTGT		
4	RV4-P1	TGTATGCTCAAACCTGCCTCTGG	1028	2031-3058
	RV4-P2	GTCTGAAGAGACATGTCAGACC		
5	RV5-P1	AAACAGGCAACACCACTGAT	1220	2473-3693
	RV5-P2	TGTAGTCACCAGCCATCTTCCA		
6	RV6-P1	GGTGCCATTAAACCGCTGCATT	996	3208-4203
	RV6-P2	CCAACTCCTCCACAACAAGATG		
7	RV7-P1	GACTTATGGATGGAACATGGGTCG	1039	4075-5113
	RV7-P2	CACCGTTAGTCACTGGAAGTCT		
8	RV8-P1	AGAGACAGGGAGGAAAGTGT	1005	4799-5803
	RV8-P2	GGGGAAGACTTGGAATAGAAATGGGC		
9	RV9-P1	GACAGACAACCTGCTCTAGGTCT	1004	5612-6615
	RV9-P2	GGTAGGACTTGTCTATCACC		
10	RV10-P1	CCGTGAACAGTTTAGTCCAGAG	1098	6331-7428
	RV10-P2	TTCCGATGAGGTCTGATCTGTC		
11	RV11-P1	CCCGACGAA GACTTGATAATTGG	959	7011-7969
	RV11-P2	AAGTCCCTCATCGGCTTGATCA		
12	RV12-P1	GGGCTGATTATCAAGAAGG	1047	7737-8783
	RV12-P2	CAACATCTCAGAAGGGTG		
13	RV13-P1	GCCGGGATTGATACAAAACCTC	1108	8546-9653
	RV13-P2	CTTGCCGTATATGTTGACAGGG		
14	RV14-P1	CACTGGCACCTTCGATGTAACA	893	9351-10243
	RV14-P2	ACTTCTTGATCCACCCAC		
15	RV15-P1	GCGAGAGTATGAGAGCTAACCT	1009	10093-11101
	RV15-P2	AGGCTCATCTCTCGAAGGGTGG		
16	RV16-P1	CCTCATCATTTGTGATGCAG	1140	10793-11932
	RV16-P2	TCGGGATCCACGCTTAACAA		

<sup>a</sup>“P1” for the forward primer and “P2” for the reverse primer; <sup>b</sup>The positions of the primers are based on the sequence of PV strain (GenBank accession no. M13215).

### RNA isolation and reverse transcription

Total RNA was extracted from brains of mice infected with the CYN1009D strain using TRIzol reagent (Invitrogen, Carlsbad, CA). Single-strand cDNAs were synthesized by reverse transcription using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Briefly, 32  $\mu$ L of total RNA was heated at 60°C for 10min, then quickly chilled on ice for at least 2 min, and then transferred to a reaction tube of Ready-To-Go You-Prime First-Strand Beads with 1 $\mu$ L of random primer pd(N)<sub>6</sub> (0.2 g/mL) (TaKaRa, Japan). After incubation at 37°C for 60 min, the synthesized cDNA product was used for PCR.

### PCR and sequencing

16 overlapping fragments were amplified and the primers are shown in Table 1. The cycling parameters were one cycle at 94°C for 3min for an initial denaturation, then 35 cycles at 94°C for 30 s, 52-57°C for 30 s, and 72°C for 80 s, and a final extension at 72°C for 10 min. The PCR products were identified on 1% Agarose (Invitrogen, USA) gel and visualized by ethidium bromide staining under UV illumination with 2 kb DNA markers (TaKaRa, Japan). All of the PCR products of the expected ED PROOF size were then excised from the agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Germany), following the manufacturer's instructions. The purified products were then directly sequenced commercially (Shanghai Sangon Biological Engineering, China).

### Amplification for 3' end and 5' end of RABV genome

To determine the sequence at the 3' end of RABV genome, PCR amplification was carried out according

to the previous method<sup>[14]</sup>. Briefly, 4  $\mu$ L (80 pmol/L) 5' phosphorylated DNA oligonucleotide 3RACE primer P (5'-P-GTCGATCACGCGATCGAACGGTCGCTGAG-3')<sup>[12]</sup>, 4  $\mu$ L RNA, 5  $\mu$ L T4 RNA Ligase Buffer, 3 $\mu$ L 0.1% BSA, 1  $\mu$ L T4 RNA Ligase (50 U/L), and 36  $\mu$ L DEPC treated water were successively added in the tube, and then incubated at 12°C for 16 h, 75% ethanol was used to precipitate the ligation product and subsequently 10  $\mu$ L DEPC treated water was added. 20 pmol oligonucleotide 3RACE inner primer (5'-CAGCGACCGTTCGATCGC-3') was used for cDNA synthesis. 3RACE inner primer and 3GSP (5'-CGACCATCATCAGGATCAAG-3') were to generate an amplicon of 290 bp. To determine the PCR product at the 5' end of RABV, a 5' Full RACE Kit was used in the present study according to the manufacturer's instructions. The primers were as follows: 5' GSP1: 5'-CGATGAAGCAGGTTATTCGAGGGA-3', 5'GSP2: 5'-CGATCTCTAGCTTGAGTCTGTC-3'.

### Sequence alignment and analysis

The resulting sequences were assembled and manually checked using the ATGC program version 4 (Genetyx Co., Tokyo, Japan). The ClustalX version 1.83 was used for multiple alignments of the sequences. DNASTar (version 5.01) was used to translate the gene sequences and to determine the percentage identities and similarity scores. Phylogenetic trees were constructed with MEGA, version 5. The background data for the rabies virus in this study are shown in Table 2.

## RESULTS

### The genomic organization of the CYN1009D isolate

The full length of the CYN1009D genome (GenBank

Table 2. The genome sequences of rabies viruses used in this study

Accession no.	Strain	Country	Year	Length	Host
EU293121	8743THA	Thailand	1983	11923	Human
EU293111	8764THA	Thailand	1983	11925	Human
AF499686	SRV9	China	2004	11928	
FJ959397	CTN-181	China	1956	11925	Human
EU643590	HN10	China	2006	11923	Human
GU358653	GX4	China	1994	11922	Dog
GU345748	SH06	China	2006	11924	Dog
FJ866836	FJ009	China	2008	11924	Dog
FJ866835	FJ008	China	2008	11924	Dog
FJ712193	D01	China	2008	11925	Dog
FJ712195	F02	China	2008	11923	Ferrt
AB085828	HEP-Flury	America	1940	11615	Human
EF206707	ERA	America		11931	
EF542830	RV-97	Russia		11932	
AY705373	SHBRV-18	USA	1983	11923	bat
EF437215	NNV-RAB-H	India	2006	11928	Human
AB044824	Nishigahara	Japan		11926	
AB128149	Ni-CE	Japan		11926	
GQ918139	CVS-11			11927	
M13215	PV	France		11932	
M31046	SAD B19	America		11928	

accession no. JQ730682) strain is 11923 nt, the genomic organization of CYN1009D, which is typical of all previously characterized RABVs, is as follows: 3' leader region (1-58), N gene (59-1483), P gene (1486-2474), M gene (2480-3282), G gene (3288-5354), L gene (5379-11853) and a trailer region (11854-11923). All five genes are initiated with AACA and terminated with poly (A)<sub>7</sub>. The coding sequences (CDS) of each of the five structural genes are located as follows: N gene 1353nt (71-1423), P gene 894nt (1514-2407), M gene 609nt (2495-3103), G gene 1575nt (3315-4889), L gene 6387nt (5406-11792); The monocistrons are separated by intergenic regions of 2, 5, 5 and 24 nucleotides respectively.

#### Structural proteins' features of CYN1009D

The N gene of the CYN1009D strain encodes a

protein of 450 amino acids residues, which are identical to the lengths of other strains in China. In comparison to other wild strains in China, the antigen site I (358-367) and antigen site III (313-367) of CYN1009D are completely conserved, variation was only detected at one position in the antigen site IV (359-383) Thr<sub>375</sub>→Met<sub>375</sub>. Furthermore, CYN1009D is different from other strains in China at five sites: Val<sub>111</sub>, Val<sub>128</sub>, Ala<sub>135</sub>, Val<sub>379</sub> and Ala<sub>426</sub>, Thr<sub>42</sub>, which are identical to those in SRV9, GX4, HN10 and CTN-181, but different from those in FJ008, FJ009, D01 and SH06. Among them, the hydrophilic amino acid Ser(S) at site 135, 426 is replaced by hydrophobic amino acid Ala(A), other variation points are between the same type of amino acid variation. Other structural and functions sites are all conserved.

PP is the structural protein with the most significant

variations in the rabies virus. The P gene of CYN1009D strain encodes a protein of 297 amino acids residues, which is identical to other strains in China in its length. Amino acids from 1-19 encode the synergetic factor of LP which participates in transcription and replication of viral RNA. It is the major site for the action of PP and LP<sup>[1]</sup> and is highly conservative in CYN1009D. The amino acids from 143 to 148 are the site of PP and the light chain LC8 of the cytoplasmic dynein, and variation was not detected in this region in CYN1009D strain and they were all DKSTQT<sup>[8]</sup>. The amino acids FSKKYKFP from 209 to 216 are major areas for the interaction between PP and NP<sup>[7]</sup>, and this region is also highly conservative in CYN1009D. Amino acids at 63~64 as well as 162, 210 and 271 have specific serine sites for protein kinase phosphorylation, which can phosphorylate PP under the effects of protein kinases, and they are major phosphorylation sites in the P gene<sup>[6]</sup>. In comparison to other strains in China, CYN1009D is conservative in amino acids at 210 and 271, but variations were detected at 63-64 and 162. Furthermore, there are more than 20 variation sites in CYN1009D.

MP is a protein that has completely identical length in all of the genotypes of rabies viruses, and The M gene of CYN1009D strain encodes a protein of 202 amino acid residues. The PPXY domain playing an important role in viral budding (35-38aa) is highly conserved. The special domain at 35~38 in MP of CYN1009D PPxY is PPEY. MP can negatively control viral transcription, but its regulatory sites during viral transcription and replication are not identical to those during viral assembly and budding<sup>[5]</sup>, and the amino acid at position 58 in the site of negative control on

transcription was E in CYN1009D, which is identical to the strains in China except SRV9 (R).

The G gene of CYN1009D strain encodes a protein of 524 amino acids residues, which is identical to other strains in China. GP is the only glycosylated protein among the structural proteins in the rabies virus, and the sites and number of glycosylation in different strains are different<sup>[22]</sup>. The glycosylation motif of the G protein is the Asn-X-Thr/Thr motif, while CYN1009D has only two common glycosylation sites: Asn<sub>37</sub> and Asn<sub>319</sub>. Among other strains in China, position 247 in SRV9 and CTN-181 is NET and it is DET/DEI in other strains, which is different from that in CYN1009D (DEA). The neutralizing antigen site I (231), II (34-42 and 198-200), positions III (330-338) and a (342-343) are conserved. Amino acids at 333, 336, 339 and 357 having the closest correlation to antigenicity<sup>[19]</sup> are conserved. In comparison to the wild strains in China, the transmembrane region of GP shows significant variations (440-461), the five amino acids are completely different from those in the wild strains in China, and two amino acids are the same as those in some strains.

The L gene of CYN1009D strain encodes a protein of 2128 amino acids residues. There are six conservative regions in the structure of LP<sup>[15,17]</sup> (which is determined by the positions of amino acids in PV strain): I (232-423), II (504-607), III (608-831), IV (889-1060), V (1090-1326) and VI (1673-1747); the short sequences (528-DLGDLPD-534 and 582-DALTMD-588) which are similar to the binding domains for magnesium ion (DXXXXXD)<sup>[21]</sup> in the conservative II region are highly conserved; Asp<sub>1706</sub> is extremely conserved in rabies viruses and this site

may affect the methyltransferase activity of L protein, and it is also conservative in CYN1009D. The unique GDNQ motif for forming phosphodiester bond in the rabies virus can be found in the conservative sequence III region of LP, and it is also termed as the RdRp active center motif. Any change in this element may lead to complete loss of polymerase activity [11], and this motif is absolutely conserved in all of the rabies virus strains used for comparison.

Excepting only one amino acid which was lost in SRV9 among the strains in China, the other strains are all composed of 2128 amino acids. The differences are found at the starting position of LP (Fig. 3), SRV9 starts with an M, while the other strains all start with two continuous M, but CYN1009D starts with ML, which is different from other strains in China. However, L and M are both nonpolar amino acids.

**Intergenic region analysis**

The composition of nucleotides in the intergenic regions of rabies viruses differs significantly. It can be found from the analysis in Table 3 that the N-P intergenic regions are all CT except that it is CG in the FJ009 street strain and TT in the F02 street strain. The P-M intergenic regions and the M-G intergenic regions for most strains are CAGGC and CTATT, while the third nucleotide in the M-G intergenic regions in CYN1009D, F02, GX4, CTN-181 and HN10 show a

CYN1009D : MLID  
 FJ008 : .M.  
 FJ009 : .M..  
 HN10 : .M..  
 SH06 : .M..  
 D01 : .M..  
 CTN-181 : .M..  
 F02 : .M..  
 GX4 : .M..  
 SRV9 : -ML.

Fig.3. Comparison in amino acid sequences at the starting positions of LP in the rabies virus strains in China.

variation from A→T. The G-L intergenic region is the longest and shows the most significant variation.

**Nucleotide sequence analysis for the terminal regulatory sequence**

3'UTR and 5'UTR in the RABV genome plays regulatory roles in viral transcription and replication [4]. The 3' UTR of CYN1009D strain is composed of 58 nucleotides and its 5'UTR is composed of 70 nucleotides. Among them 11 nucleotides in 3'UTR and 5'UTR of CYN1009D strain are conservative and completely reverse complementary.

**Phylogenetic analysis**

Whole-genome phylogeny (Fig. 4) using nucleotide sequences reveals that CYN1009D and the street strains in China are distributed in two different clusters, while it is allocated in the same cluster with 8764THA and 8743THA from Thailand, indicating that they have a closer genetic relationship.

Table 3. Intergenic region analysis between CYN1009D and other stains in China.

Strain	N-P	P-M	M-G	G-L
CYN1009D	CT	CAGGC	CTTTT	CTGTGGAACAGAAGGACAATTAGT
SH06	. .	. . . . .	. . A . .	. . . . A . . . . T A . . . . A . . . . C . . . C
D01	. .	. . . . .	. . A . .	. . . . A . . . . T A . . . . A . . . . C . . . C
FJ009	. G	. . . . .	. . A . G	. . . . A . . . . T A . . . . A . . . . C . . . C
FJ008	. .	. . . . .	. . A . .	. . . . A . . . . T A . . . . A . . . . C . . . C
SRV9	. .	. . . . .	. . A . .	. . A T . A . . . . T . . . . . A . . . . C . G . C
F02	T .	. . . . .	. . . . .	. . A . A . . . . T . . . . A . . . . C . . . C
GX4	. .	. . . . .	. . . . .	. . A . . . . . T . . . . . . . . . C . . . C
CTN-181	. .	. . . T . .	. . . . .	. . A . A . . G . T . . . . . . . . . C . . . C
HN10	. .	. . . . G	. . . . .	. . A . A . . . . . . . . . . . . . . C . . . C

GP is the major determinative factor for pathogenesis of viruses and the G gene is significantly affected by selection pressure, which is always related to highly synonymous mutation and is an ideal target for investigations on the natural evolution of viruses [10, 24]. The present study selected the G genes from other strains from Yunnan province, strains from other provinces adjacent to Yunnan and representative strains from other Asian countries to construct the cladogram. The results show that the strains in Yunnan province can be divided into two clusters, some strains in Yunnan were allocated in the same cluster with the strains from Guangxi, Fujian, Zhejiang and Chongqing, indicating that they have a relatively close genetic relationship; while the CYN1009D strain and another strain from Yunnan are allocated in the same cluster with the strains from Southeast Asian countries such as Burma, Laos, Vietnam, Cambodia, Thailand and Malaysia. The strains from other Asian countries are allocated in another two clusters.

### DISCUSSION

The complete genome of CYN1009D is composed of 11923 nt and it belongs to genotype I, which did not significantly differ from the complete genomic sequences of other rabies viruses published in GenBank, but is identical in length to a previously determined strain by our laboratory HN10. Among the genotype I of *Lyssavirus*, the lengths of complete genomes are normally between 11923 nt and 11928 nt<sup>[2]</sup>, a strain with 11922 nt (GX4) has also been reported in China, but the significance for the length of the complete genome for the characteristics of viruses is still unknown.

In the comparisons in major functional sites between CYN1009D and other strains, particularly the antigen sites of NP and GP, mutations in some amino acids were detected. However, mutations were not detected in key positions, such as Ser89 of NP which is related to the capability for viral transcription and replication, PPxY of MP which plays an important role in viral budding, positions 330 and 333 of GP which are directly related to the virulence of viruses, which also indirectly implies the important roles of these sites. Thr<sub>375</sub> in the antigen site IV (359-383) of NP is highly conservative among the vaccine strains and the wild strains, but it is mutated into Met in the CYN1009D strain. Position 375 is a minor phosphorylation site and the phosphorylation at this site is affected by the phosphorylation at position 389. Once position 389 was dephosphorylated, position 375 was also dephosphorylated accordingly<sup>[9]</sup>, and the effects for the mutation at this site on the strain still need further study.

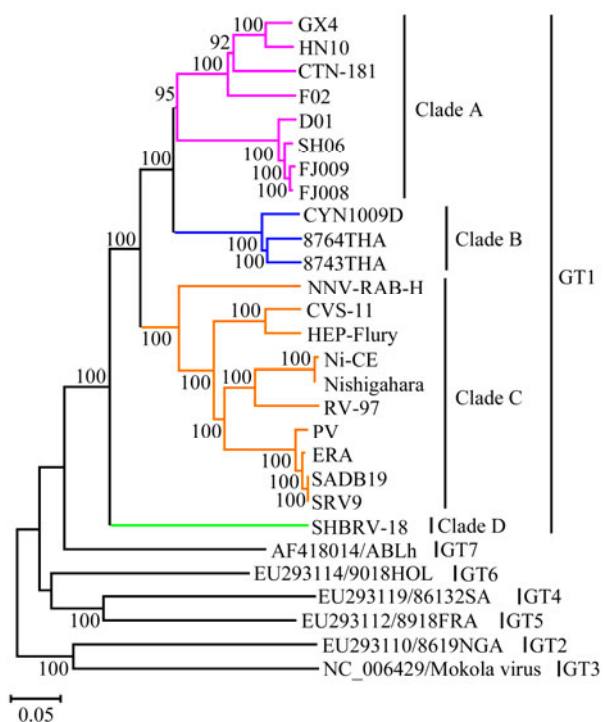


Fig. 4. The phylogenetic analysis for the complete genomic sequences of rabies viruses.



two Met residues and they were relatively independent in phyletic evolution, which is mainly found in wild strains in China, and it has not been reported in the strains from France, India, Brazil and Thailand <sup>[13]</sup>. The second amino acid in CYN1009D in the present study is mutated into a nonpolar amino acid L. The functions of this mutation in virus evolution and transmission are still unclear now and more information can be discovered after further analysis is carried out on LP in rabies viruses from other areas.

The phylogenetic analysis of the G gene shows that the geographical characteristic of rabies virus in Yunnan is that the specimens from the same province (the same geographical location) tend to evolve to the same evolutionary cluster or sub-cluster. Some strains in Yunnan show relatively close genetic relationship to those in adjacent provinces such as Guangxi and Sichuan, while the CYN1009D strain shows relatively close genetic relationship to the strains from Southeast Asian countries particularly the strains from Burma, indicating that the epidemic strains of rabies virus in Yunnan have a close genetic relationship to the strains from adjacent provinces and adjacent countries in Southeast Asia. It is estimated that the prevalence of rabies in Yunnan is related to the distribution and expanding of rabies viruses, and the possibility for expanding from other countries can not be ruled out in addition to the general expansion from different provinces. The geographical expansion of the Chinese strains in China and simultaneous expansion in adjacent countries may affect the prevalence and distribution of rabies in China and adjacent countries. Therefore, the prevention and control rabies at borderland areas should be reinforced simultaneously when efforts are made to control rabies in China,

which is under control. These are essential procedures for the effective prevention and control of rabies all over the world.

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