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Evidence for inter- and intra-clade recombinations in rabies virus

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ABSTRACT

Homologous recombination is considered rare in negative-strand RNA viruses and has not been reported for rabies virus. In this study, full-length genomes of 44 rabies virus strains were analyzed for potential homologous recombination events. Phylogenetic analysis classified these strains into three clades. By applying six different recombination detection methods, one inter-clade and one intra-clade potential recombination events were identified with high confidence values. Software-predicted recombination break points of the two events were all located within the polymerase gene. This report presents the first evidence suggesting the possibility of homologous recombination in rabies virus, which could provide valuable insights for understanding the diversity and evolution of rabies virus as well as other negative-strand RNA viruses.

1. Introduction

Rabies is a fatal disease caused by infection of rabies virus that is usually transmitted to humans through bites by rabid animals. Rabies has been threatening the lives of mankind for more than 4000 years (Knobel et al., 2005). Despite the existence of effective pre- and post-exposure prophylactic measures, over 55,000 people die of the disease annually, mainly in developing Asian and African countries (Knobel et al., 2005; Schnell et al., 2010).

Rabies virus is the prototype virus of the *lyssavirus* genus of the *Rhabdoviridae* family. It has a single-stranded, negative-sense RNA genome of ~12 k nucleotides, which consists of five genes encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L), respectively (Tordo and Kouknetzoff, 1993; Wunner et al., 1988). Domestic and wild mammals such as dogs, cats, foxes, bats and ferret badgers are major natural hosts and vectors of rabies virus infection (Blancou et al., 1986; Tang et al., 2005; Zhang et al., 2009a, 2005).

Recently, a phylogenetic analysis based on the viral G gene of Chinese isolates and representative sequences from other countries classified rabies virus into three clades: clade I consists of strains isolated in China and Southeast Asia, clade II is distributed throughout the globe, whereas clade III is found only in bats and raccoons in the Americas (Zhang et al., 2009b).

Recombination is one important way for viruses to gain genetic diversity, but homologous recombination appears to be rare in non-segmented negative-strand RNA viruses (Chare et al., 2003). In the current study, we investigated the possibility of homologous recombination in rabies virus by analyzing publicly available complete genomic sequences. One inter-clade event and one intra-clade events were identified with high confidence values. Significance of this finding and possible underlying mechanisms are discussed.

2. Materials and methods

Only complete genomic sequences of rabies virus strains were included for analysis in this study, as it has been reported that phylogenetic analyses based on complete genomes are more reliable than those based on individual genes (Boore and Staton, 2002; Gubser et al., 2004; Herniou et al., 2001). Complete rabies virus genomic sequences available in GenBank by June 2010, excluding patented sequences, were aligned using ClustalW (Thompson et al., 1994). The alignment was then manually adjusted to correct open reading frames and different clones from the same experimental sample with 100% sequence identity were treated as a single entity. A total of 44 non-redundant full-length genomes were obtained (listed in Table 1) and re-aligned to generate a phylogenetic tree using the maximum likelihood method in PhyML 3.0 (Guindon et al., 2010). MODELTEST version 3.7 (Posada and Crandall, 1998) was adopted to select the best-fit model of nucleotide substitution and identified the general time-reversible model with

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Table 1
List of the complete genomic sequences retrieved from GenBank used in this study.

Accession No.	Strain name	Source	Place of isolation	References
AB009663	RC-HL	Vaccine strain	Japan	Ito et al. (2001)
AB044824	Nishigahara	Vaccine strain	Japan	Ito et al. (2001)
AB085828	HEP-Flury		Japan	Inoue et al. (2003)
AB362483	BR-Pfx1	Fox	Japan	Mochizuki et al. (2009)
AY705373	SHBRV-18	Silver-haired bat	USA	Faber et al. (2004)
AY956319	1	Human	Germany	GenBank
DQ875050	MRV	Mouse	Henan, China	Qian and Zhao (2008)
DQ875051	DRV	Sika deer	Jilin, China	Qian and Zhao (2008)
EF206707	ERA	Vaccine strain	Germany	Geue et al. (2008)
EF206708	Lysvulpen	Vaccine strain	Germany	Geue et al. (2008)
EF206709	Fuchsoral	Vaccine strain	Germany	Geue et al. (2008)
EF206715	SAD P5/88	Vaccine strain	Germany	Geue et al. (2008)
EF206718	SAD1-3670var2	Vaccine strain	Germany	Geue et al. (2008)
EF206720	Sanafox	Vaccine strain	Germany	Geue et al. (2008)
EF437215	NNV-RAB-H	Human	India	GenBank
EF542830	RV-97	Vaccine strain	Russia	Metlin et al. (2008)
EF564174	CTN181	Human	Shandong, China	GenBank
EU293111	8764THA	Human	Thailand	Delmas et al. (2008)
EU293113	9001FRA	Dog	Guyana	Delmas et al. (2008)
EU293115	9147FRA	Fox	France	Delmas et al. (2008)
EU293116	9704ARG	Free-tailed bat	Argentina	Delmas et al. (2008)
EU293121	8743THA	Human	Thailand	Delmas et al. (2008)
EU311738	RRV ON-99-2	Raccoon	Canada	Szanto et al. (2008)
EU549783	BD06	Dog	China	GenBank
EU643590	HN10	Human	Hunan, China	Ming et al. (2009)
EU877067	SAD B19-4th	Fox	Germany	Beckert et al. (2009)
EU877068	SAD B19-1st	Fox	Germany	Beckert et al. (2009)
FJ712193	D01	Dog	China	Lei et al. (2010)
FJ712194	D02	Dog	China	Lei et al. (2010)
FJ712195	F02	Chinese ferret badger	China	Lei et al. (2010)
FJ712196	F04	Chinese ferret badger	China	Lei et al. (2010)
FJ866835	FJ008	Dog	Fujian, China	GenBank
FJ866836	FJ009	Dog	Fujian, China	GenBank
FJ913470	ERA-VC		China	GenBank
FJ959397	CTN-1	Human	Zibo, China	GenBank
GU345746	CQ92	Dog	Chongqing, China	Meng et al. (2010)
GU345747	J	Human	Ningxia, China	Meng et al. (2010)
GU345748	SH06	Dog	Shanghai, China	Meng et al. (2010)
GU358653	GX4	Dog	Guangxi, China	Meng et al. (2010)
GU565703	Flury-LEP	Vaccine strain	Haerbin, China	GenBank
GU565704	Flury-HEP		Haerbin, China	GenBank
GU647092	JX08-45	Chinese ferret badger	China	GenBank
M13215	PV	Vaccine strain	France	Tordo et al. (1988)
M31046	SAD B19	Vaccine strain	Germany	Conzelmann et al. (1990)
EF157976	EBLV-1	Serotine bat	Germany	Marston et al. (2007)
EF157977	EBLV-2	Bat worker	Scotland, UK	Marston et al. (2007)

rate heterogeneity among sites and invariable sites (GTR+I+G) using both the hierarchical likelihood-ratio test (hLRT) and the Akaike information criterion (AIC). The robustness of the phylogenetic tree was evaluated by performing bootstrap analysis using 100 pseudoreplicate datasets. The neighbor-joining method implemented in MEGA4 (Tamura et al., 2007) with Jukes-Cantor distances was used for confirmation. The statistical significance of the reconstructed phylogenies was estimated by bootstrap analysis with 2000 replicates.

Detection of potential recombinant sequences, identification of candidate parental sequences as well as determination of possible recombination break points were performed using Recombination Detection Program (RDP) (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BOOTSCAN (Martin et al., 2005), MaxChi (Smith, 1992), CHIMAERA (Posada and Crandall, 2001) and SISCAN (Gibbs et al., 2000) methods embedded in the RDP3 software package (Martin et al., 2010) with default parameters for each method. Only recombination events detectable by at least three different methods were taken as evidence of recombination and reconfirmed using the neighbor-joining method implemented in MEGA4 (Tamura et al., 2007). The statistical significance of the reconstructed phylogenies was tested by bootstrap analysis with 2000 replicates.

3. Results and discussion

A total of 44 non-redundant full-length rabies virus genomic sequences were obtained and a phylogenetic tree using the maximum likelihood method consisting of three distinct clades was built with high bootstrap values (Fig. S1). A phylogenetic tree generated using neighbor-joining method showed nearly identical topology (data not shown). The topology of the phylogenetic tree and geographic distribution of the clades are highly similar with the results of a previous report analyzing the sequences of viral G gene (Zhang et al., 2009b).

Sequences of the 44 viral genomes were subjected to recombination analysis using the RDP3 software package as described in Materials and Methods. Recombination signals detected with significant *P* values by all six different recombination detection methods in RDP3 are listed in Table 2. The three potential recombination events with highest degree of confidence were further analyzed and confirmed using BOOTSCAN plot (Martin et al., 2005) and neighbor-joining method (2000 replicates, JC model) in MEGA4 (Tamura et al., 2007).

As illustrated in Fig. 1, Event I is a recombination between the lineages represented by the isolate GX4 from a dog (GU358653, Guangxi, China, Meng et al., 2010) of clade I as the major parent

Table 2
The characteristics of detectable rabies virus recombination events.

Event number	Breakpoint positions		Recombinant	Major parent	Minor parent	Detection methods and *P*-values					
	Begin	End				RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan
I	7841	8343	HN10	GX4	DRV	1.97E-67	3.28E-67	9.20E-70	8.07E-15	3.83E-15	1.06E-18
II	11,308	11,654	F04	JX08-45	SH06	7.72E-27	1.09E-25	5.97E-27	4.83E-07	3.81E-06	1.49E-03
III	11,308	11,657	F02	JX08-45	SH06	7.72E-27	1.09E-25	5.97E-27	4.83E-07	3.81E-06	1.49E-03
IV	19*	185	F04	JX08-45	FJ009	5.77E-24	1.36E-22	4.24E-24	2.58E-09	1.95E-09	5.40E-06
V	19*	409	F02	JX08-45	FJ009	5.77E-24	1.36E-22	4.24E-24	2.58E-09	1.95E-09	5.40E-06
VI	4946	4993	HEP-Flury	Flury-HEP	MRV	6.17E-11	4.66E-10	9.76E-12	NS	NS	5.47E-03
VII	21*	174	DRV	RC-HL	Unknown(RV-97)	5.73E-18	NS	1.85E-17	2.70E-07	3.91E-09	5.44E-07
VIII	10*	1118	DRV	Flury-LEP	Unknown(FJ009)	1.15E-03	1.91E-05	1.43E-07	NS	NS	NS
IX	0*	92	MRV	Flury-LEP	Unknown(FJ009)	1.15E-03	1.91E-05	1.43E-07	NS	NS	NS

NS, no significant *P*-value was recorded for this recombination event using this method.
* The actual breakpoint position is undetermined.

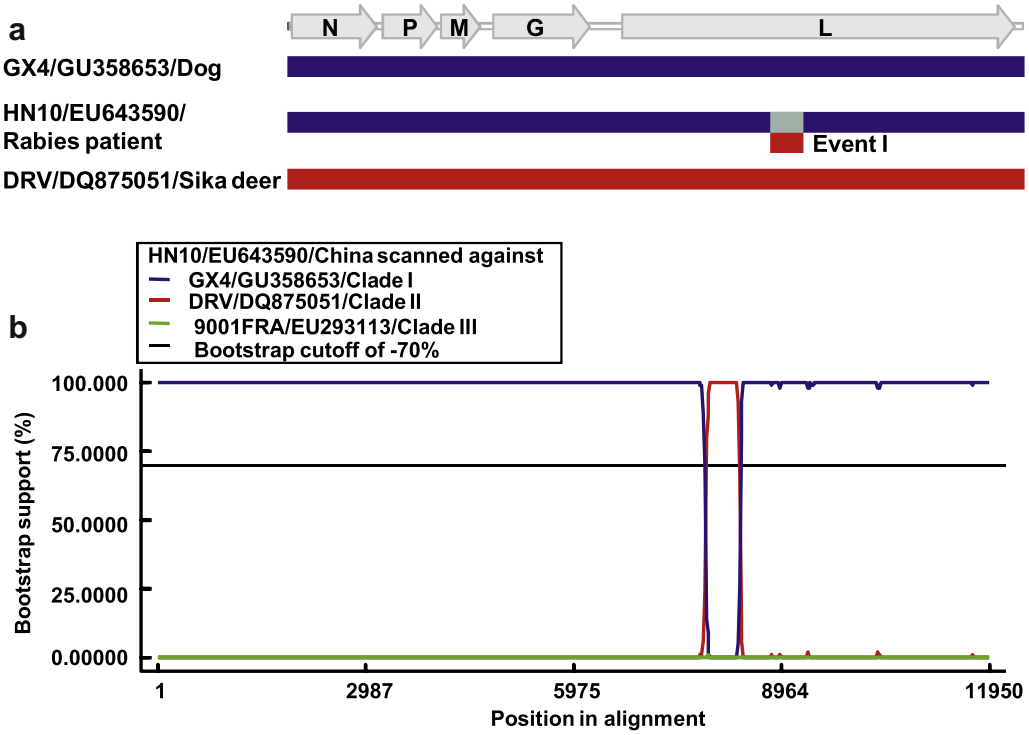

Fig. 1. Identification of recombination event in rabies virus. (a) One inter-clade recombination event between clade I and clade II was identified among analyzed rabies virus isolates using the RDP3 software package. (b) BOOTSCAN analysis of recombination event I. HN10 (EU643590) was analyzed against GX4 (GU358653) and DRV (DQ875051) which represent parental strains. 9001FRA (EU293113) was used as outgroup. BOOTSCAN evidence for the recombination origin on the basis of pairwise distance was modeled with a window size of 200, step size of 20 and 100 bootstrap replicates.

and the isolate DRV from a sika deer (DQ875051, Jilin, China, Qian and Zhao, 2008) of clade II as the minor parent, which led to the recombinant isolate HN10 from a rabies patient (EU643590, Hunan, China, Ming et al., 2009). In this event, a GX4-like virus exchanged a segment (7841–8343 nt) of the polymerase region with a DRV-like virus (Fig. 2).

Events II and III are intra-clade recombination events, which occurred between the lineages represented by the clade I isolates JX08-45 from Chinese ferret badgers (GU647092, China) and SH06 from dogs (GU345748, China), respectively, giving rise to two recombinant isolates F02 (FJ712195, China) and F04 (FJ712196, China) from Chinese ferret badgers (Fig. 3). In these events, a JX08-45-like virus exchanged the segments (11,308–11,657 and 11,308–11,654 nt) of the polymerase region with a SH06-like virus (Fig. 4). The fact that the exchanged segments in Events II and III differ only by 3 nucleotides at one termini suggests that F02 and F04 might be two diverging descendents from a common recombinant ancestor strain, which was derived from a single recombination event between a JX08-45-like virus and a SH06-like virus involving the identified region of polymerase gene.

Due to the size of its genome, genomic sequencing of rabies virus usually involves amplifying and sequencing of multiple, overlapping segments that are later aligned in software. Errors like sample mix-up in this process could result in sequences falsely identified as recombinant in phylogenetic analysis. To examine such a possibility, we evaluated the available sequencing information on the three identified recombinants in this study. Sequences of the putative exchanged segment of the recombinant HN10 in Event I were obtained by aligning three overlapping amplicons (Ming et al., 2009), whereas sequences of the putative exchanged segment of the recombinant F02 and F04 were derived from a single amplicon containing little adjoining sequences on either side

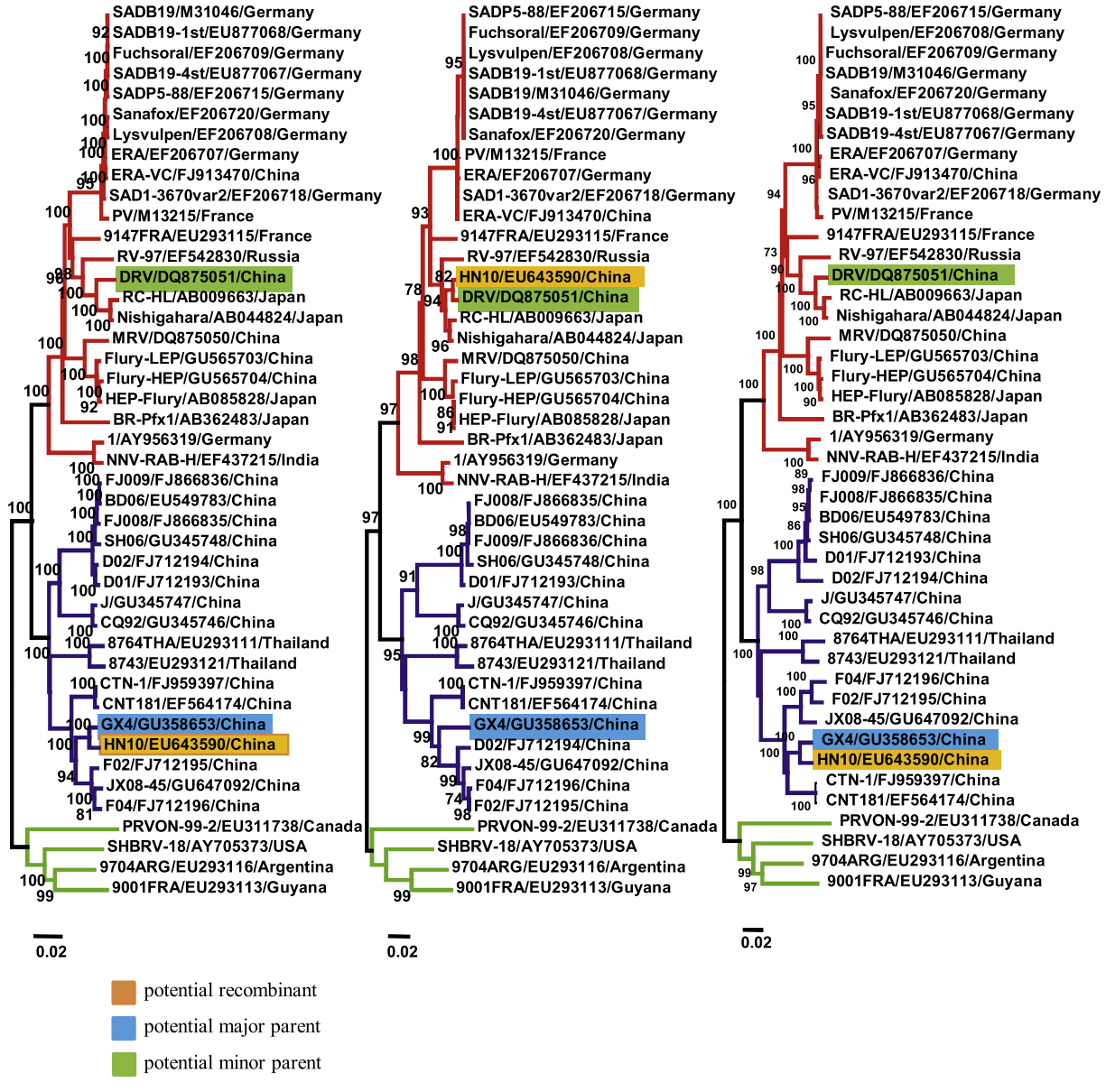

Fig. 2. Phylogenetic analysis of the recombination event I. Sequences exchanged in the recombination event (7841–8343 nt) and the rest of the corresponding genomes were separately analyzed using neighbor-joining method and Jukes-Cantor model in MEGA4. Groupings were tested by bootstrapping 2000 replicates and support percentages greater than 70% are marked at the corresponding nodes.

(Lei et al., 2010). It is therefore not very likely for HN10 to be a false recombinant caused by experimental errors, but more caution is needed in regarding F02 and F04.

Recombination is a widespread mechanism of genetic variation and increasingly viewed as important for driving genetic diversity in RNA viruses (Worobey and Holmes, 1999). Compared to other RNA viruses, negative-strand RNA viruses appear to have relatively low rates of recombination (Chare et al., 2003; Schierup et al., 2005). Definitive evidence of rabies virus recombination has not been reported prior to this report, although Chare and colleagues did manage to find sequence variation patterns suggestive of recombination (Chare et al., 2003).

Co-infection of the same host by different virus strains is a prerequisite for homologous recombination. So far, there does not seem to have been any report of rabies virus co-infection of natural hosts in the literature. However, the existence of incubation period in rabies virus infection (Charlton et al., 1997) suggests that co-infection should be considered a realistic possibility. In co-infected hosts, co-infection or super-infection by different virus strains of the same target cell is also required for homologous recombination. Using mutants with detectable phenotypic traits and distinguishable sequence mutations, Spann and colleagues were able to prove experimentally that co-infection and recombination could happen to the non-segmented negative-strand RNA virus human respiratory syncytial virus (RSV) (Spann et al., 2003). It would be interesting to apply similar strategies to rabies and other non-segmented negative-strand RNA viruses to gain insights into the possibility of co-infection and/or super-infection resulting in recombination.

Recombination of non-segmented negative-strand RNA virus is generally believed to occur through 'copy-choice' replication: the viral RNA-dependent RNA polymerase (RDRP) switches to a different template midway during replication, generating chimeric or recombinant replication products (Chare et al., 2003). Theoretically,

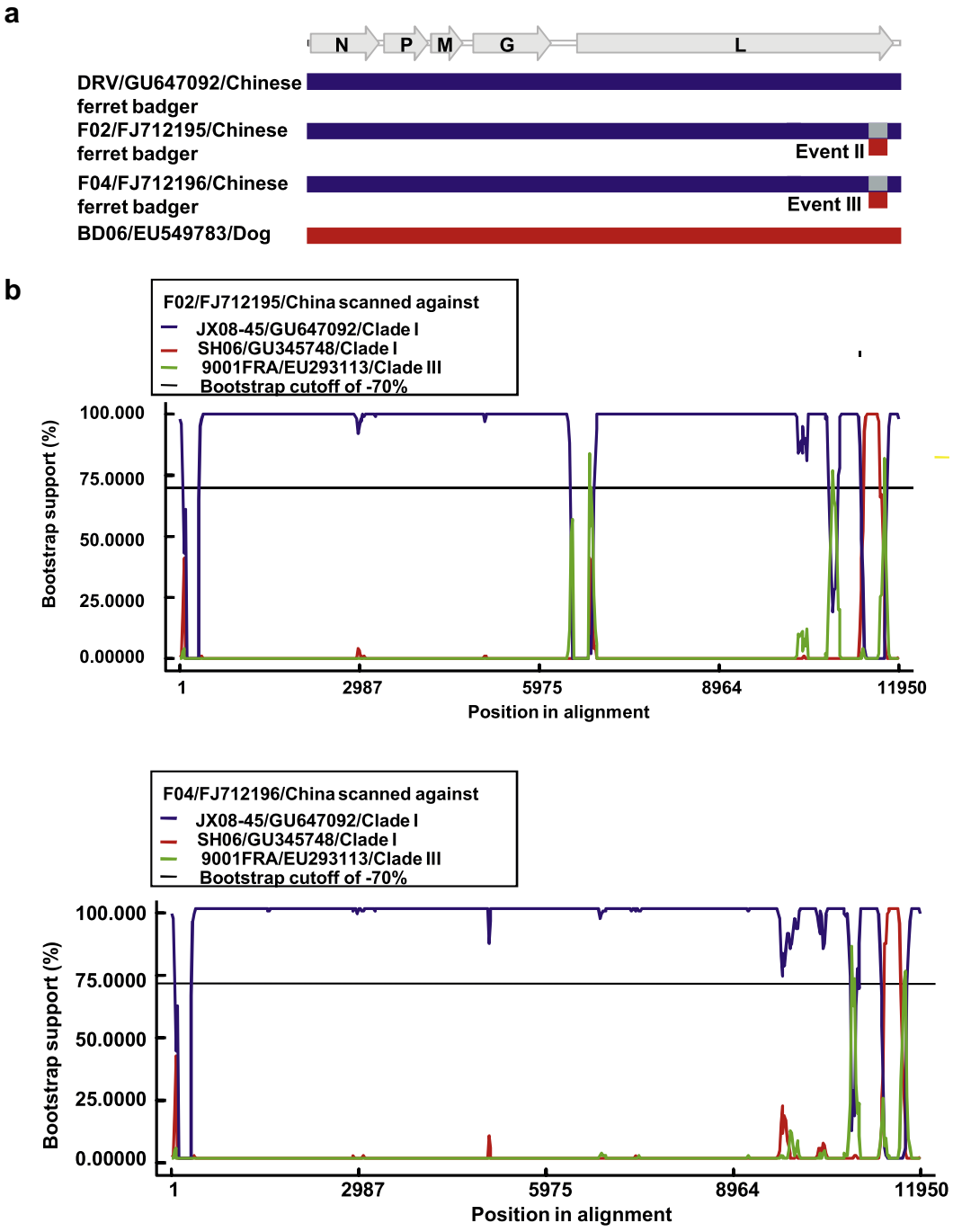

Fig. 3. Identification of recombination events in rabies virus. (a) Two intra-clade recombination events in clade I were identified among analyzed rabies virus isolates using the RDP3 software package. (b) BOOTSCAN analysis of recombination event II and III. F02 (FJ712195) and F04 (FJ712196) were, respectively analyzed against JX08.45 (GU647092) and SH06 (GU345748) which represent parental strains. 9001FRA (EU293113) was used as outgroup. BOOTSCAN evidence for the recombination origin on the basis of pairwise distance was modeled with a window size of 200, step size of 20 and 100 bootstrap replicates.

in co-infected target cells, template switch could take place when parent genome is being transcribed to produce positive-strand cRNA, or when pre-genomic cRNA is being replicated to generate progeny negative-strand viral genome. Both these processes are catalyzed by the same viral RDRP.

Recombination provides virus with a mechanism for rapid adaptation to different host environments. The recombination events discussed in this work only involve the viral polymerase gene and are unlikely to offer any advantages for binding receptors or evading host immune defense. Instead, changes in viral RDRP might help rabies virus transcribe and replicate more efficiently in a new host where cellular factors essential for viral life cycle could be significantly different. It is possible that continuous monitoring and sequence analysis of rabies virus may reveal recombination events involving other viral genes or non-coding sequences.

Viral recombination has important implications for detection and vaccination. Given the high mortality rate of rabies virus infection, evidence presented in this work suggests that more rigorous

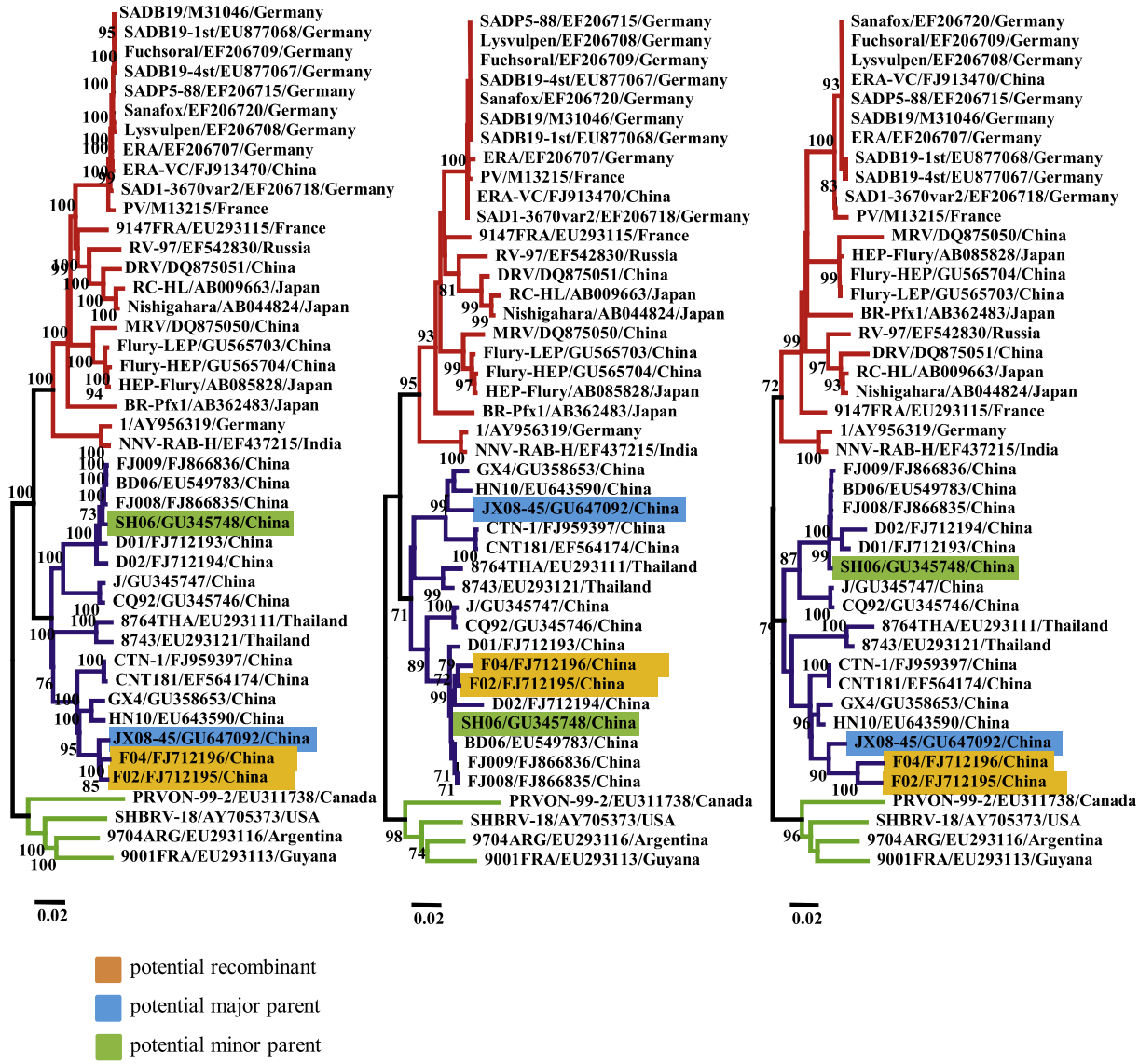

Fig. 4. Phylogenetic analysis of the recombination event II and III. Sequences exchanged in the recombination event (11,308–11,657 and 11,308–11,654 nt) and the rest of the corresponding genomes were separately analyzed using neighbor-joining method and Jukes–Cantor model in MEGA4. Groupings were tested by bootstrapping 2000 replicates and support percentages greater than 70% are marked at the corresponding nodes.

experimental and bioinformatic efforts are warranted to investigate rabies virus recombination both in its natural hosts and in laboratory conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.08.031.

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