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SHORT REPORT

Identification and characterization of a novel natural recombinant avian leucosis virus from Chinese indigenous chicken flock

Xiongyan Liang[1] · Yufang Gu[1] · Xueyang Chen[1] · Tuofan Li[1] · Yulong Gao[2] · Xiaomei Wang[2] · Chun Fang[1] · Shouguo Fang[1] · Yuying Yang[1]

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Abstract

Avian leukosis virus (ALV) caused tremendous economic losses to poultry industry all over the world, especially in China. One natural recombinant ALV strain, designated as HB2015032, was isolated from indigenous chickens with neoplastic diseases in Hubei, China. The complete proviral genome of HB2015032 is 7703 bp in length. Sequence analysis showed that the Env of HB2015032 exhibited 99.3% similarity with that of a ALV subgroup K (ALV-K) isolate JS11C1 at amino acid level. Phylogenetic analysis revealed that both gp85 and gp37 of HB2015032 were clustered in the same branch with JS11C1 and other ALV-K strains isolated from Chinese indigenous chickens in recent years. However, the *pol* gene, the 3′ untranslated region (3′ UTR), and the 3′ long terminal repeat (3′ LTR) of HB2015032 were more closely related to ALV-J prototype HPRS-103, and clustered in the same branch with ALV-J strains. Furthermore, the *pol* gene of HB2015032 contained a premature stop codon that resulted in a truncated Pol protein with 22 amino acid residues missing, which was a unique feature of the pol gene of ALV-J. 3′UTR of HB2015032 containing entire DR1, E element and U3. E element of HB2015032 contained one base deletion, which resulted in a c-Ets-1 binding site. In addition, U3 region of HB2015032 contains most of the transcription regulatory elements of ALV-J, including two CAAT boxes, Y boxes, CArG boxes, PRE boxes, NFAP-1 boxes, and one TATA box. These results suggest that isolate HB2015032 was a novel recombinant ALV-K containing the ALV-K *env* gene and the ALV-J backbone and exhibiting high pathogenicity.

Keywords Avian leukosis virus · ALV-K · ALV-J · Recombinant

Introduction

Avian leukosis virus (ALV) is a member of the genus Alpha-retroviruses in the family Retroviridae, which could be divided into different subgroups according to the characteristics of viral envelope protein [1, 2]. Basing on the transmission model, ALV could also be divided into exogenous and endogenous viruses [3–5]. The exogenous ALV contains ALV-A, ALV-B, ALV-C, ALV-D, and ALV-J, among which ALV-A, ALV-B, and ALV-J were the main subgroups that causing avian neoplastic diseases in the world [1–6].

During the past two decades, avian leukosis have caused tremendous economic losses to poultry industry in China [7–10], although the Nationwide Eradication Program (NEP) of ALV was carried out in the primary chicken flocks since 2008 [11]. Up to now, ALV-J has caused myelocytoma and hemangioma remained prevalence in China [12, 13]. Moreover, there were many new avian leukosis viruses isolated from different flocks in recent years, which were identified

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Xiongyan Liang and Yufang Gu have contributed equally to this work.

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✉ Yuying Yang
yangyycn@yangtzeu.edu.cn

[1] School of Animal Science, Yangtze University, No.88, Jingmi Road, Jingzhou 434025, China

[2] Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 678, Haping Road, Harbin, China

as recombinant viruses from different avian leukosis virus subgroups [11, 14, 15]. The most impressive was the report showed that several ALV strains with a novel *env* gene were isolated from Chinese local chickens [16–21]. These isolates were divided into ALV subgroup K (ALV-K) by Cui et al., as they exhibited no or low pathogenicity and their *gp*85 were low homology in comparison with that of other subgroups [17, 18]. So far, this new subgroup of ALV strains were only isolated from the local breeds in East Asia [16–21].

In this study, we isolated a novel natural recombinant ALV designated as HB2015032 from indigenous chicken flock in Hubei, China. To understand the molecular characteristics of this isolate, the complete proviral genome of HB2015032 was sequenced and compared with the representative isolates of other ALV subgroups. Our data showed that HB2015032 contained ALV-K-like *env* gene but with the ALV-J-like backbone and exhibited high pathogenicity, which might be considered as a novel ALV recombinant.

Materials and methods

Background of the animal epidemic materials

In November 2015, five 20-week-old ill-chickens which suspected to be avian leukosis from a commercial indigenous chicken farm in Hubei, China, were submitted to our laboratory for further diagnosis based on multi-PCR and ELISA [22, 23].

Virus isolation, identification, and proviral genome amplification

Virus isolation was performed on chicken fibroblasts DF-1. The cells were seeded into six-well plate with Dulbecco's modified eagle medium (DMEM, Invitrogen, Shanghai, China) contained 10% fetal bovine serum (FBS, Gemini, USA). Then cells were co-cultured with filtered liver homogenates from the most serious case (named HB2015032) at 70% confluent and incubated for 1 h at 37 °C. Then cells were continuously cultured with DMEM contained 1% FBS at 37 °C in an incubator with 5% CO_2 for 7 days for one passage. Following three blind passages, the culture supernatant was harvested for ALV group-specific antigen p27 detection using ALV Antigen Test Kit (IDEXX, Yuangheng, Being, China) as described previously [24].

To obtain the complete proviral genome, genomic DNA of p27 positive DF-1 cells were extracted with Tissue Genomic DNA Purification Kit (GeneRay Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. Then the proviral genome were amplified as three overlapped fragments with the primer sequences listed as following, ALV-F1, TGTAGTSTTATGCAATRCTCTT; ALV-R1, GCATGGGAATTCCCCCTCCTAT; ALV-F2, GAATTCCCATGCGAAAATCT; ALV-R2, TCATCCTTTTGGGTGATGT; ALV-F3, GACCCCCGGTTAARATACGAAT; ALV-R3, TGAAGCCATCCGCTTCATGCAGGT . PCR was conducted in a 50-μL volume according to the manufacturer's instruction of Phanta Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd, Nanjing, China). The PCR products were purified with Takara MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Takara, Dalian, China) and then cloned into the pTOPO-Blunt Simple vector (Aidlab, Biotech Co., Ltd, Beijing, China) for sequencing.

Sequencing, multiple alignments, and phylogenetic analysis

The genomic fragments were sequenced by TSINGKE Co., Ltd (Wuhan, China) and assembled with SeqMan program of Lasergene (version 7.10). Complete genome was submitted to GenBank and reference ALV genomes were downloaded from GenBank for alignments and phylogenetic analysis (Table 1). Multiple alignments and phylogenetic trees were established with Megalign program of Lasergene (version 7.10) and MEGA (version 6.0), respectively.

Results and discussion

ELISA data showed that DF-1 cells inoculated with filtered liver homogenates following by three blind passages (Case No. HB2015032) were ALV-p27 positive, indicating that the liver homogenates contained exogenous ALV.

The genome of HB2015032 was assembled from sequences of three overlapped PCR fragments. The full-length provirus genome of HB2015032 was 7703 bp in length (GenBank Accession No. KY581580) and contained a typical genetic structure of type C retrovirus as "LTR-leader-*gag*-*pol*-*env*-LTR". The *env*-LTR fragment was 2696 bp in length and the *env* region was highly similar to ALV-K isolates. Three main genes *gag*, *pol*, and *env* of the isolate were 2103 bp, 2619 bp, and 1806 bp in length, respectively.

Further, sequence analysis showed that the Env of HB2015032 exhibited amino acid identity of 94.8–99.3% with indicated ALV-K strains isolated from Chinese indigenous chickens, whereas only 46.4–88.8% amino acid identity to other ALV subgroups. And gp85 of HB2015032 showed 92.8–99.4% amino acid homology with indicated ALV-K isolates. Phylogenetic analysis of gp85 showed that HB2015032 and other ALV-K isolates were clustered in the same branch (Fig. 1). Similar result was observed in phylogenetic analysis base on gp37 (Supplementary Fig. 1). These data suggested that the isolate HB2015032 belonged to ALV-K. But chicken infection and virus titer test showed

Table 1 ALV reference strains in this study

Isolate	Subgroup	Origin	Accession no.	Isolate	Subgroup	Origin	Accession no.
RSA	A	France	M3780	SD09DP03	J	China	Jn62487
MAV-1	A	USA	L10922	SD09DP04	J	China	HQ634808
TW-3577	A	China	HM582657	GD1109	J	China	JX254901
RAV-2	B	USA	M14902	sdau1001	J	China	JN389517
RSV-S-R-B	B	USA	AF052428	WB11098(J)	J	China	JX848322
GX14FF03	B	China	KU923579	JS09GY3	J	China	GU982308
RSV-Prague	C	USA	J02342	JS09GY6	J	China	GU9823108
RSV-S-R-D	D	USA	D10652	SCAU-HN06	J	China	HQ900844
ev-1	E	USA	AY013303	GX14ZS14	J	China	KX037423
ev-3	E	USA	AY013304	TW-3593	K	China	HM582685
ev-6	E	USA	AY013305	JS11C1	K	China	KF746200
SD0501	E	China	EF467236	GDFX0601	K	China	KP686142
HPRS-103	J	UK	Z46390	GDFX0602	K	China	KP686143
ADOL-7501	J	USA	AY027920	GDFX0603	K	China	KP686144
SDAU1701	J	China	KY980657	GD14LZ	K	China	KU605774
GX14YL03	J	China	KR025484	JS14CZ01	K	China	KY490695
GX14HG01	J	China	KU997685	JS14CZ02	K	China	KY490696
GX14HG04	J	China	KX058878	HB2015032	K	China	KY581580
GX14ZS14	J	China	KX037423	SDAUAK-11	K	China	KY773911
GX14LT07	J	China	KX034517	SDAUAK-12	K	China	KY773912
GX14YYA1	J	China	MF461280	SDAUAK-13	K	China	KY773923
sdau1002	J	China	JN389518	ALV-K-env-J	K	China	MK638922

that HB2015032 exhibited tumorigenicity and higher replication ability (Supplementary Fig. 2).

The *gag* gene was highly conserved among different subgroups of ALV, and the *gag* gene of HB2015032 was same as that of other ALV. However, the *pol* gene of HB2015032 contained a mutation as ALV-J, which was premature stop at the position of 5345 bp in HB2015032 genome (corresponding to the position of 5346 bp in HPRS-103 genome). This mutation resulted in a truncated Pol which was 22 amino acid residues shorter than that of the other ALV subgroups (Supplementary Fig. 3). This premature stop of *pol* was unlikely to affect either the mature Pol proteins or virus growth [25], which was different from the ALV-K SDAUAK-11 in which mutations in the *pol* region is affected on the reverse transcription activity and replication [26]. Importantly, the premature stop of *pol* was one of the unique features of ALV-J [27, 28]. And phylogenetic analysis based on the Pol showed that HB2015032 was clustered in the group of ALV-J (Fig. 2). These data suggested that the Pol of HB2015032 might derive from virus of ALV-J.

Moreover, the 3′UTR+LTR of HB2015032 was highly similar to that of ALV-J prototype HPRS-103(94.0%) and isolates, such as WB11908(J) (96.2%) and SD09DP04 (95.6%). Phylogenetic tree based on the 3′UTR+LTR showed that HB2015032 and the twenty-one ALV-J representative strains were clustered in the same branch (Fig. 3). It was quite different from the ALV-K strains (GDFX0601, GDFX0602, GDFX0603, JS14CZ02, TW3593, JS14CZ01, and GD14LZ) isolated from Chinese indigenous chicken flocks, which exhibited no or low pathogenicity (Fig. 3). The 3′UTR+LTR of those low virulent ALV-K isolates were clustered in the same branch with ALV-E [16, 19, 20]. Further analysis showed that the 3′UTR+LTR of HB2015032 included entire DR1, E element, U3, R, and U5 regions, but without the non-functional redundant transmembrane (rTM) region (Supplementary Fig. 4), which was virtually identical to ALV-J ADOL-7501 [9]. As we known, E element (also named as XSR) was function as an enhancer which has been found only in ALV-J and Rous sarcoma virus [27, 29]. The absence of rTM suggests that 3′UTR of HB2015032 might be from AVL-J of layer flocks, since most of ALV-J strains isolated from layer-type chickens had the deletion of rTM [30, 31]. In particular, E element of HB2015032 contained 1-bp deletion at the position of 7251 (corresponding to the position of 7388 bp in HPRS-103) (Supplementary Fig. 4). The 1-bp deletion resulted in a binding site of c-Ets-1, which is associated with the differentiation of vascular endothelial cell [9, 29]. In ALV-J, the binding site of c-Ets-1 is associated with hemangioma of layer chickens [9]. Moreover, the U3 of HB2015032 contained several transcriptional regulation elements as ALV-J, including two CAAT boxes, two Y boxes, two CArG boxes, two PRE boxes, one NFAP-1 box, and one TATA box (Supplementary Fig. 4), which are associated with the replication capacity of ALV [32]. These

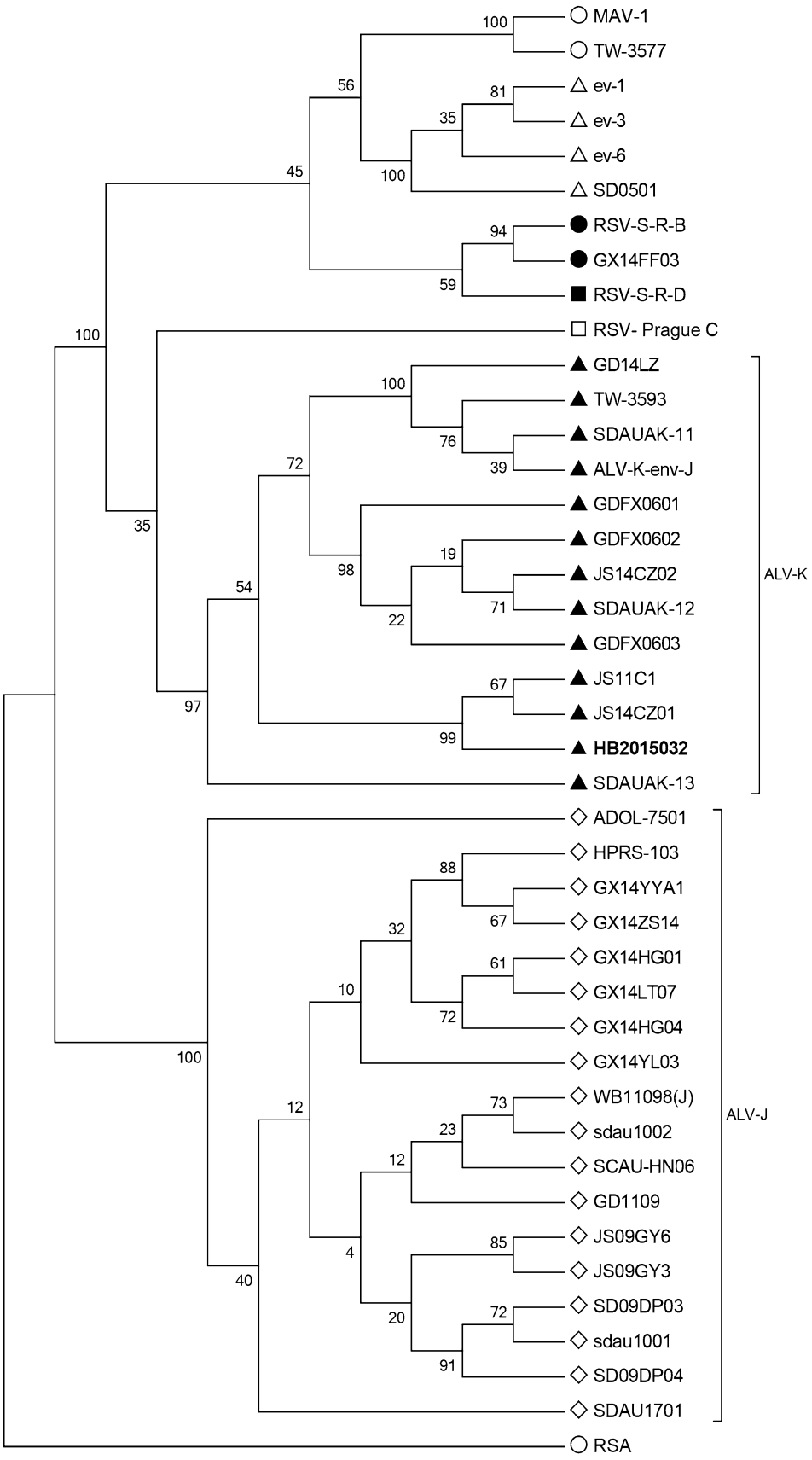

Fig. 1 Phylogenetic tree analysis of *env* gene encoding SU protein of different ALV strains. The tree was constructed using the neighbor-joining method (1000 bootstraps) with MEGA 6.0. Hollow circle indicates ALV-A, solid circle indicates ALV-B, hollow square indicates ALV-C, solid square indicates ALV-D, hollow triangle indicates ALV-E, solid triangle indicates ALV-K, and hollow diamond indicates ALV-J

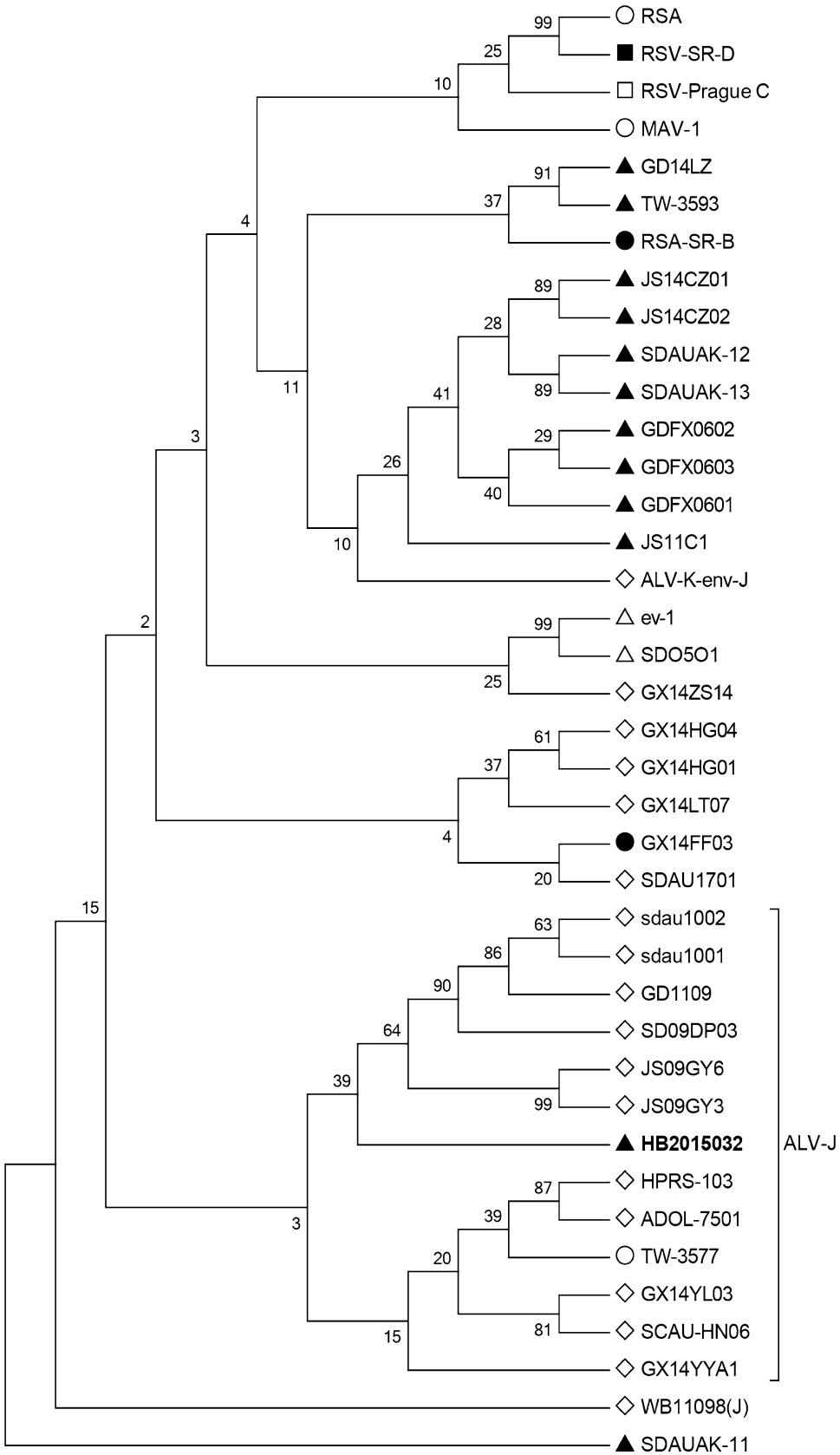

Fig. 2 Phylogenetic tree analysis of Pol of different ALV strains. The trees were constructed using the neighbor-joining method (1000 bootstraps) with MEGA 6.0. Hollow circle indicates ALV-A, solid circle indicates ALV-B, hollow square indicates ALV-C, solid square indicates ALV-D, hollow triangle indicates ALV-E, solid triangle indicates ALV-K, and hollow diamond indicates ALV-J

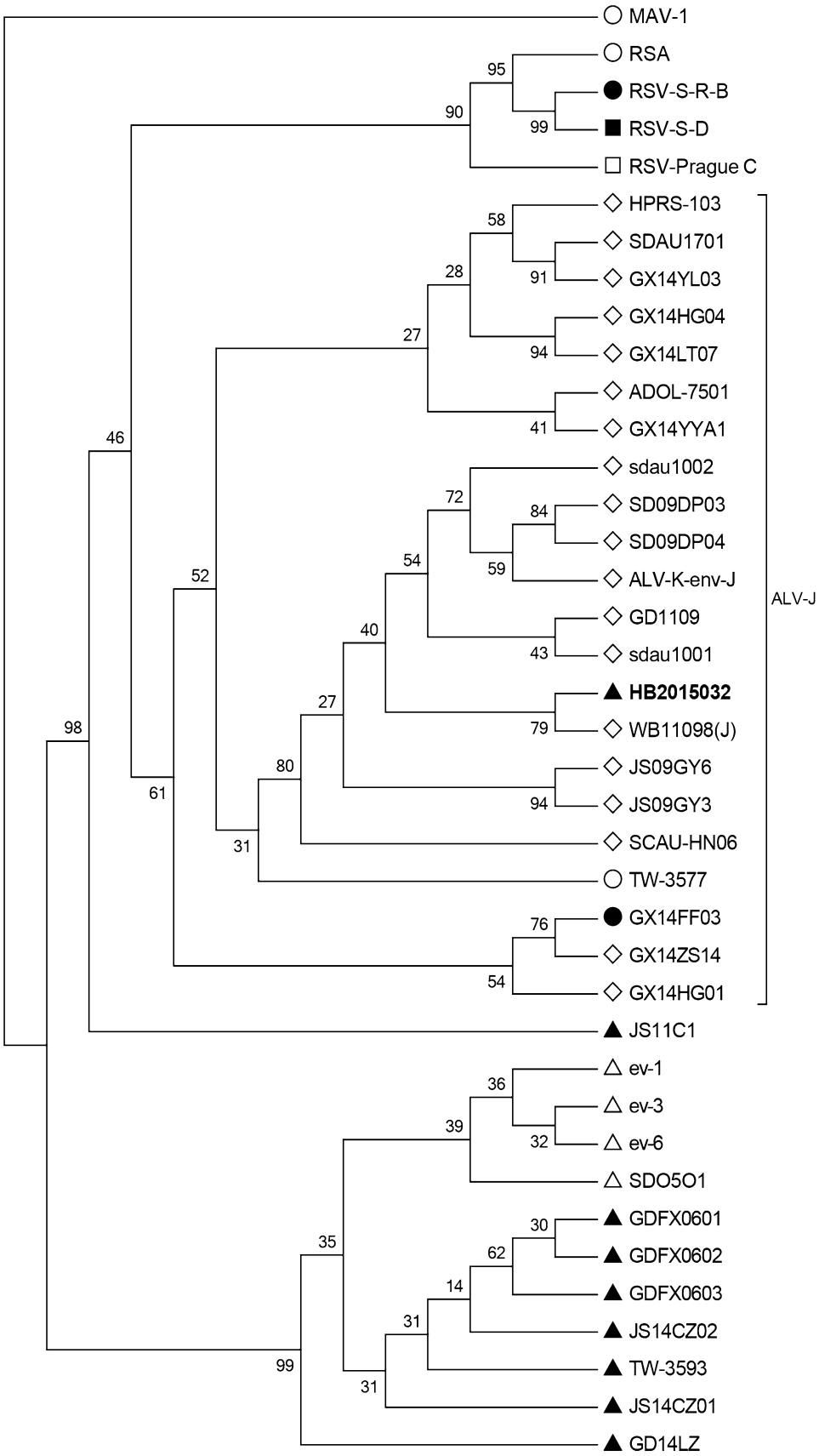

Fig. 3 Phylogenetic tree analysis of 3′UTR + LTR. The trees were constructed based on the nucleic acid sequences of 3'UTR+LTR by the neighbor-joining method (1000 bootstraps) with MEGA 6.0. Hollow circle indicates ALV-A, solid circle indicates ALV-B, hollow square indicates ALV-C, solid square indicates ALV-D, hollow triangle indicates ALV-E, solid triangle indicates ALV-K, and hollow diamond indicates ALV-J

elements might explain the enhancement of the replication ability of HB2015032.

In summary, we isolated a novel natural recombinant ALV-K from a commercial Huainan indigenous chicken flock in Hubei province, China. Genomic analysis demonstrated that the isolate HB2015032 contained ALV-J backbone with ALV-K *env* that exhibited high pathogenicity to chickens. The genome of HB2015032 was similar to that of the artificial recombinant ALV-K-env-J [33] with the differences observed in the Pol (Supplementary Fig. 3). This is the first report on ALV-K naturally recombined with ALV-J. In addition, the finding also reminds us to pay more attention to the new recombinant ALV-K in ALV eradication program in the future.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was declared by the authors.

Ethical approval All applicable international, national, and institutional guidelines for the use and care of animals were followed.

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