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Genetic Diversity of Ranaviruses in Amphibians in China: 10 New Isolates and Their Implications

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ABSTRACT

Ranaviruses can cause amphibian mass deaths and contribute to global ecological instability. In this study, 10 ranavirus strains isolated from *Rana dybowskii* and *Rana amurensis* Boulenger across three geographic locations in Northeastern China were studied through PCR, cloning, sequencing, restriction endonuclease analysis (REA), and phylogenetic analysis. Although the isolates were highly homologous to frog virus 3, genetic differences were observed among Northeastern Chinese isolates, as well as between these isolates and those from different regions worldwide. Different ratios of substitutions and/or missense mutations were observed through REA at several loci of each isolate, which resulted in shortening or lengthening of the restriction-enzyme cutting sites of *Fnu4H I*. The cutting sites of other endonucleases (*Hinc II, Acc I*, and *PflM I*) were not affected by the existing mutations. Changes in the REA spectra of the Northeastern Chinese isolates were observed, thereby indicating the high level of genetic diversity of ranaviruses in this region. Nevertheless, further research must be performed over a broader area to confirm the diversity of ranaviruses. This study recommends the inclusion of ranaviruses in pathogen surveillance worldwide.

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XZ and XW designed the study, executed the experimental work, analyzed the data and wrote the article.

Key words:

Ranaviruses, genetic diversity, identification methods, REA, amphibians.

INTRODUCTION

Global declines in amphibian populations are one of the most pressing and enigmatic environmental problems of the late 20th century (Daszak *et al.*, 1999). Approximately 30% (1895 species) of amphibians worldwide are threatened with extinction (*i.e.*, listed in the International Union for Conservation of Nature (IUCN) Red List Categories as Vulnerable, Endangered, or Critically Endangered) (IUCN, 2000). As a principal cause of the notable increase in recent amphibian die-offs, emerging infectious diseases have been the focus of scientific investigations (Rachowicz *et al.*, 2006). Among various pathogens examined, ranaviruses have shown to be of great importance and are linked to amphibian mass mortality events throughout several regions worldwide (Daszak *et al.*, 1999; Grayfer *et al.*, 2012).

Ranaviruses, which belong to the family Iridoviridae, have been detected in a wide range of amphibian populations in Europe (Martel *et al.*, 2012), Australia (Speare and Smith, 1992), South America (Fox *et al.*, 2006), North America (Souza *et al.*, 2012) and Asia (He *et al.*, 2002). As ranaviruses can be highly virulent, they cause systemic infections in amphibians (Daszak *et al.*, 1999). Ranaviral disease in frogs causes mortality rates of nearly 100% in *Bufo marinus* (cane toad) tadpoles and 40% in *Lithobates catesbeianus* (North American bullfrog) metamorphs (Daszak *et al.*, 1999; Green *et al.*, 2002). Ranavirus invades liver, kidneys, and digestive tract and causes hemorrhage in skeletal muscles (Hyatt *et al.*, 1998). Robert *et al.* (2005) found that frog virus 3 (FV3) infections in *Xenopus* spp. have a strong tropism for the kidney, specifically the proximal tubular epithelium. Ultimately, in many cases, animals die with a striking edema indicative of renal dysfunction.

The recent and frequent outbreaks of ranavirosis in China have impelled the need to investigate susceptibility to ranavirus infection, variations in the expression of disease among amphibian species and developmental stages, and the effects of recombination in the emergence of strains with increased virulence. Moreover, analysis of the genetic basis of pathogen virulence and host susceptibility in free-ranging populations of amphibians has been an important endeavor.

China, which is the third largest country in the world (9.6 million km^2 land mass) (Li *et al.*, 2011; Xi'An Map Press, 2005), comprises a wide array of climatic regions and vegetation types and provides habitat (Xie *et al.*, 2007) for 399 amphibian species (6.28% of global species abundance). A few historical records are available

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with regard to population fluctuations of amphibian species in China (Wei *et al.*, 2010), and research on the presence and effects of amphibian diseases is also scarce. Currently, studies of iridovirus infections on amphibians in farmed and wild populations in the country have been increasing. For instance, *Rana grylio* virus and tiger frog virus were isolated from cultured frogs in Southern China (Zhang *et al.*, 2001; He *et al.*, 2002). Other types of infections were also noted in *Andrias davidianus* (giant salamanders) in Northwestern China (Geng *et al.*, 2011) and in free-range *Rana dybowskii* (Dybowski's frog) in Northern China (Xu *et al.*, 2010).

The diversity of ranaviruses in China has been rarely investigated. Meanwhile, ranavirus isolates were generally obtained from farmed species and widely separated regions; as such, scholars are restrained from identifying the level of ranavirus diversity in China.

In this study, 10 isolates from wild *Rana dybowskii* and *Rana amurensis* Boulenger were subjected to multiple genetic analyses to determine their characteristics and assess their diversity. Results may encourage researchers to invest more effort in understanding the whole picture of ranaviruses and their diversity in China.

MATERIALS AND METHODS

Viral isolates

Ten ranavirus isolates obtained from two native wild amphibian species in Northeastern China were identified and preserved in our laboratory. Eight of these strains were isolated from *R. dybowskii* across three geographic locations, including Heihe (isolates D107, D108, and D211), Hebei (isolates A105, A106, and b1040303), and Dongfanghong (isolates B3S1 and B3S2). The remaining two strains were obtained from *R. amurensis* Boulenger (Siberian wood frog, isolates b2030303 and b2090303) from Hebei.

The reference sequences used for analysis included the following: the type species of the genus ranavirus frog virus 3 **(**FV3, Accession number: U36913) and Gutapo virus (GV, Accession number: AF157649) from the Americas; *Rana tigrina* ranavirus (ZS, Accession number: AY033630) and soft-shelled turtle iridovirus (SITVZS, Accession number: EU627010) from China; Bohle virus (BIV, Accession number: AY187046) and epizootic hematopoietic necrosis virus (EHNV, Accession number: AY187045) from Australia; Wamena virus (WV, Marsh *et al.*, 2002) from Indonesia; European catfish virus (ECV, Accession number: AF157659) and European sheatfish virus (ESV, Accession number: AF157679) from Europe; and *Rana catesbeiana* virus (JP, Accession number: AB474588) from Japan.

Characteristic analysis of isolates from Northeastern China

Cloning and sequencing

DNA template was extracted from the liver tissues of 10 native anurans from Northeastern China, which were positive for ranaviral disease, by using the standard protocol of Tianamp genomic DNA kit for animal tissues (Tiangen, China). Amplification was conducted using four primers:
M151 (5′ -AACCCGGCTTTCGGGCAGC A-3′),
M152 (5′-CGGGGCG GGGTTGATGAGAT-3′),
M153 (5′-ATGACCGTCGCCCTC ATCAC-3′), and
M154 (5′-CCATCGAGCCGTTCATG ATG-3′)
(Marsh *et al.*, 2002) for each virus to achieve two PCR products of different lengths: MCP-1 (321 bp, primers M151 and M152) and MCP-2 (625 bp, primers M153 and M154). Thermocycling conditions were identical for all samples (35 cycles of 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; extension of 72°C for 5 min; and cooling at 4°C). The products were purified using the Gel Extraction Kit (HaiGene Co. Ltd., China).

Cloning was performed according to the instructions of pMD®18-T vector kit (TaKaRa Biotechnology Co., Ltd., China). The clones were sent to three different companies, namely, Sangon Biotechnology Co. Ltd. (Shanghai, China), Invitrogen Trade Co. Ltd. (Shanghai, China), and TaKaRa Biotechnology Co. Ltd. (Dalian, China) for commercial sequencing.

Restriction endonuclease analysis (REA)

PCR-REA was performed as recommended by OIE according to the instructions of the manufacturer for *PflM I* (New England Biolabs, Co., Ltd.) on MCP-1 (321 bp) and for *Hinc II*, *Acc I*, and *Fnu4H I* (TaKaRa, China) on MCP-2 (625 bp).

Phylogenetic analysis

Phylogenetic analyses were performed on 10 MCP-2 (625 bp) target viral fragments from native Chinese anurans and 10 global reference sequences (Viral isolates of this study) by using the Molecular Evolutionary Genetics Analysis (MEGA) database. Phylogenetic relationships were inferred through neighbor-joining distance by using MEGA 4.0.2. Phylogenetic trees were drawn to scale, and the numbers at the nodes of the tree indicated bootstrap values of 1000 replicates (values below 70 were not depicted). Branch lengths were proportional to the number of amino acid substitutions, as indicated by the scale bar at the bottom.

RESULTS

Genetic diversity of isolated Ranaviruses

The MCP-1 (321 bp) and MCP-2 (625 bp)

Table I.- Mutations by substitutions in the 625 bp part of MCP in 10 novel Chinese ranavirus strains compared with FV3. Base position (refer to Fig. 1). FV3 from North America (Accession number: U36913) was used as reference.

Sample sites	Species	Sampling codes	Substitutions and base position									Mutations	
			48	118	123	188	225	351	447	521	528	573	605	Numbers of mutation	No. of missense mutation
Hebei	*Rana dybowskii*	A105	***T***	***G*⁺**	C	C	T	G	A	C	T	C	***C*⁺**	3	2
Hebei	*Rana dybowskii*	A106	***T***	***G*⁺**	C	C	T	G	A	C	T	C	G	2	1
Hebei	*Rana dybowskii*	b10403	C	***G*⁺**	C	C	***G***	***A***	A	C	T	C	G	3	1
Hebei	*Rana amurensis*	b20303	***T***	***G*⁺**	C	C	***G***	***A***	A	C	T	C	***C*⁺**	5	2
Hebei	*Rana amurensis*	b20903	***T***	***G*⁺**	C	***T*⁺**	T	G	***G***	***T*⁺**	T	***T***	***C*⁺**	7	4
Dongfh	*Rana dybowskii*	B3S1	***T***	***G*⁺**	C	C	T	G	A	C	T	C	***C*⁺**	3	2
Dongfh	*Rana dybowskii*	B3S2	***T***	***G*⁺**	C	C	T	G	A	C	T	C	***C*⁺**	3	2
Heihe	*Rana dybowskii*	D107	***T***	***G*⁺**	***T***	C	T	G	A	C	T	C	G	3	1
Heihe	*Rana dybowskii*	D108	C	***G*⁺**	C	C	T	***A***	A	C	***C***	C	***C*⁺**	4	2
Heihe	*Rana dybowskii*	D211	C	***G*⁺**	C	C	T	***A***	A	C	***C***	C	***C*⁺**	4	2

Note: Changes are labeled in italic and bold fonts. '+' indicates the occurrence of missense mutations. Circled numeric characters in the column of Code indicate phylogenetic clustering. Dongfh symbols represent Dongfanghong town.

fragments of the 10 strains from Northeastern China were cloned and separately sequenced in triplicate (*i.e.*, each clone was sequenced by three different laboratories). The results were consistent among the laboratories.

Analyses on the MCP nucleotide sequence (625 bp fragments) revealed that all 10 strains from Northeastern China were highly homologous to FV3, with the results as follows: A106 (99.68%); A105, B3S1, B3S2, and D107 (99.52%); D108, D211, and b10404 (99.36%); b20303 (99.20%); and b20903 (98.88%).

Mutations (substitutions) were observed at several loci, and some of these mutations were regarded as missense mutation (Table I). These mutations shortened or lengthened the position of restriction-enzyme cutting sites of *Fnu4H I* in all strains. However, the presence of these mutations, including substitutions and missense mutations, did not affect the restriction-enzyme cutting sites of other endonucleases (*Hinc II*, *Acc I*, and *PflM I*; Fig.1).

In reference to host infections, two strains isolated from *R. amurensis* Boulenger showed more mutations than those from *R. dybowskii* (Table I, Fig. 1).

Characteristics of isolated Ranaviruses

The REA electrophoretic spectra of ranavirus strains from native anurans in Northeastern China varied from those of known isolates.

The results obtained with the use of endonucleases *Hinc II*, *Acc I*, and *PflM I* showed that the cutting sites were identical to FV3 in 100% (10/10) of the isolates from the current study. Three isolates (D108, D211, and b10403) could not be distinguished from BIV through digestion with *Fnu4H I*. However, the remaining seven isolates (b20303, A105, B3S1, B3S2, D107, A106, and b20903) were unique and produced results that diverged from FV3 and from other reference strains (Table II); this divergence occurred as a consequence of a base mutation from C to T on position 48 (Table II, Fig. 1-A1). Furthermore, an extra mutation from C to T on position 521 in isolate b20903 was observed (Table I, Fig. 1-A2). These two mutations deleted the cutting site of *Fnu4H I* and changed the REA spectrum (Table II). By contrast, a mutation from A to G on position 118 in seven strains (b20303, A105, B3S1, B3S2, D107, A106, and b20903) increased the cutting site of *Fnu4H I* but also changed the REA spectrum (Tables I and II).

Phylogenetic analysis

According to the phylogenetic tree, the wild strains of ranaviruses in Northeastern China were highly homologous to FV3. However, genetic differences were observed among the isolates from Northeastern China, as well as between these isolates and those from other

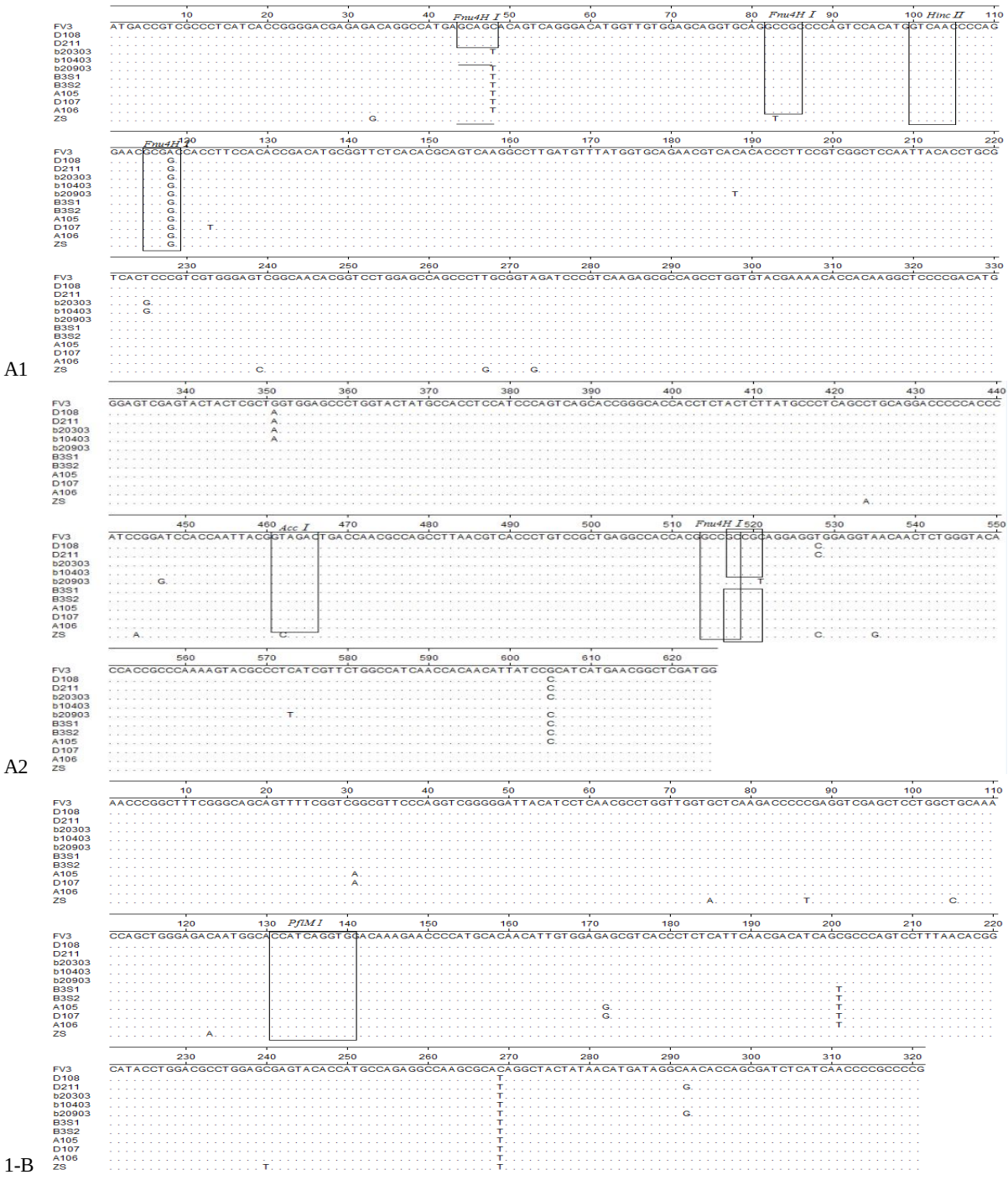

Fig. 1. Multi-sequence alignment and enzyme cutting sites of 10 novel ranavirus isolates from Northeastern China compared with isolates from Southern China (ZS) and North America (FV3). “A” is the result of sequence comparison of all 625 bp fragments. “B” is the result of sequence comparison of all 321 bp fragments. Mutation was considered to have occurred when differences were detected compared with FV3. Enzyme cutting sites of each enzyme are indicated by rectangles or underline. ZS (Accession number: AY033630) is the strain isolated by Sun Yat-sen University from China.

Table II.- Digestion spectrum of four selected restriction endonucleases applied to 10 novel Chinese isolates and FV3 (the type species) to determine conflicts with the "standard spectrum" suggested by OIE. The obtained spectrum was constrained because the possible size of the fragments from enzymatic digestion was predetermined according to the cutting sites. The Chinese isolates and FV3 showed one to several base differences in comparison with the OIE standard spectrum.

Code		Selected endonucleases			
		***PflM I* (bp)**	***Hinc* (bp)**	***Acc* (bp)**	***Fnu4H* (bp)**
A105		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
A106		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
B3S1		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
B3S2		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
D107		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
D108		137, 184	102, 523	163, 462	3, 33, 38, 45, 107, 399
D211		137, 184	102, 523	163, 462	3, 33, 38, 45, 107, 399
b10403		137, 184	102, 523	163, 462	3, 33, 38, 45, 107, 399
b20303		137, 184	102, 523	163, 462	3, 33, 83, 107, 399
b20903		137, 184	102, 523	163, 462	33, 83, 110, 399
ZS		137, 184	102, 523	625	3, 45, 71, 107, 399
JP		137, 184	102, 523	625	3, 45, 71, 107, 399
FV3	Obtained spectrum	137, 184	102, 523	163, 462	3, 33, 38, 45, 107, 399
	Standard spectrum	131, 190	100, 525	164, 461	3, 38, 44, 108, 432
BIV	Obtained pectrum	321	102, 523	625	3, 33, 38, 45, 107, 399
	Standard spectrum	321	100, 525	625	3, 33, 38, 44, 108, 399
WV	Obtained spectrum	137, 184	102, 238, 285	625	3, 45, 71, 107, 399
	Standard spectrum	131, 190	100, 240, 285	625	3, 44, 71, 108, 399
EHNV	Obtained spectrum	321	102, 138, 385	240, 385	33, 38, 45, 239, 270
	Standard spectrum	321	100, 138, 387	238, 387	33, 38, 44, 239, 271
ESV	Obtained spectrum	/	/	625	3, 9, 33，38, 45, 107, 151, 239 3, 9, 38, 44, 108, 151, 272
	Standard spectrum	/	/	625	3, 9, 38, 44, 108, 151, 272
ECV	Standard spectrum	/	/	625	3, 9, 38, 44, 108, 151, 272
GV	Standard spectrum	/	/	164, 461	3, 38, 44, 108, 432

regions worldwide, as indicated by base mutations (Fig. 1). Within Northeast China, some mutations could be commonly found in isolates from adjacent regions (Table I, Fig. 2).

DISCUSSION

In this study, high genetic diversity was detected in 10 ranavirus isolates from three different adjacent geographical locations (Hebei, Heihe, and Dongfanghong) in Heilongjiang province, China by using multiple analytical methods.

Heihe and Dongfanghong are located 382 km northwest and 138 km east of Hebei, respectively (Fig. 3A). The strains from Heihe shared common regional characteristics but also showed similarity to the strains from Hebei (Table I). Hebei is between Heihe and Dongfanghong (Fig. 3B) and may be a convergence site for various strains of ranavirus.

Sequence analyses revealed differences in enzyme cutting sites between isolates from the North American bullfrog in Japan (JP) and *Rana rugulosa* in China (ZS) (Fig. 1). We performed single digestion with four different enzymes. Digestion with *Hinc II* and *Acc I* identified JP and ZS as BIV, whereas digestion with *Fnu4H I* identified these species as WV. Meanwhile, digestion with *PflM I* identified JP and ZS as FV3.

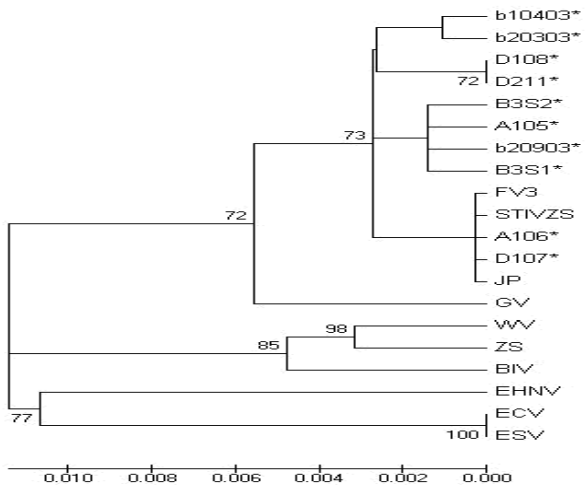

Fig. 2. Phylogenetic analysis on 625 bp MCPs of 10 novel Chinese isolates. (For information on isolates, please refer to the Materials and Methods section). Codes with stars denote isolates obtained from Northeastern China.

Considerable differences among Northeastern (present study) and Southern Chinese isolates (ZS) are summarized in Figure 2. Northeastern isolates showed the highest number of differences in nucleotide sequences compared with the isolates (ZS) obtained from *R. rugulosa* by scholars from Sun Yat-sen University. The mechanism and time of occurrence of these differences can be determined using the optimal method, which starts from the uninterrupted historical monitoring records of the development of ranaviruses in China, to outline the trend of distribution and evolution of ranaviruses. A few of these integrated data can be accessed in China.

Although increasing reports on ranaviruses in China have been available over the past 2 decades and have contributed to research hotspots in terms of geographical aspect, a number of issues still exist. First, some areas remain uncovered by surveys. When a large regional part of China or the whole country is regarded as an integrated investigation unit in an epidemiological survey, the upper hotspots are insufficient to constitute the reasonable geographical web with substantial region omission. Survey design among these reports does not agree with one another most of the time, which may impose issues regarding data reliability and identification. Second, only a few epidemiological surveys are available in China. From the aspect of geographical epidemiology, most reports provided insufficient information on distribution and spreading, which hampered the simulation of spatial and temporal dynamic course of ranaviruses and the determination of driving factors.

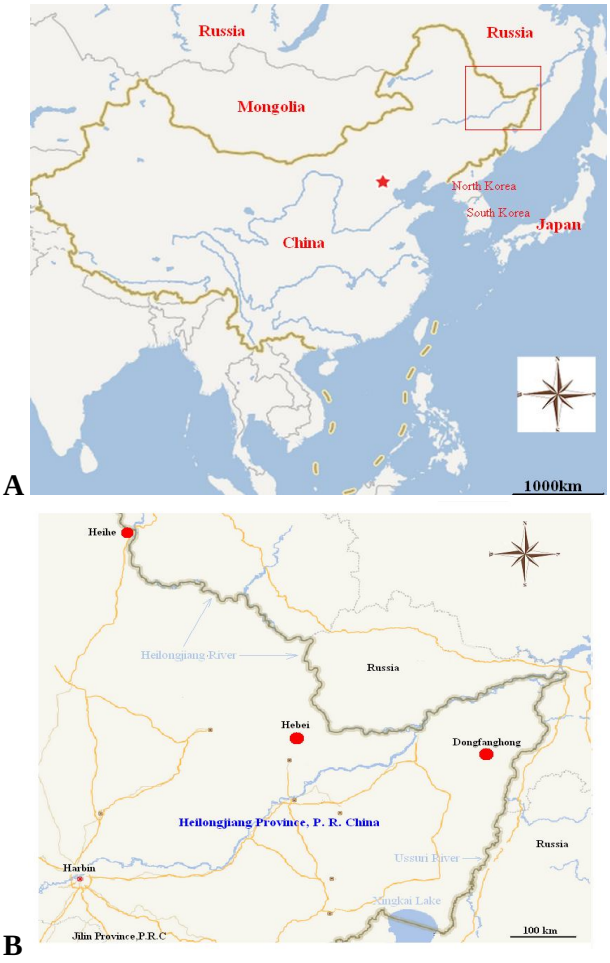

Fig. 3. Study sites. A, Map of China showing adjacent countries; the region under study is indicated by the red open rectangular box; B, Map of Eastern Heilongjiang province showing the locations of the sampling sites (red dots).

Another possible mechanism that affects changes in ranaviruses is the immune evasion strategies developed during virus-host co-evolution; these strategies inhibit the capability of the immune host to attack and complete virus replication and transmission (Huang *et al.*, 2014). This hypothesis is confirmed by the result of the present study, in which the 625 bp part of MCP of the 10 strains were highly homologous to BIV, as determined through amino acid sequence alignment (result not shown). Given the function of MCP in virion assembly, we speculate that this phenomenon is caused by evolution, rather than an accident. FV3 and BIV may exhibit the same unknown mechanism for virion assembly and/or the mechanism allowing a virus to replicate in a specific host by

inhibiting the innate and acquired immunity of a host.

Artificial breeding of frogs begun in mainland China in the late 1950s, and North American bullfrogs were introduced from Japan by Shanghai Fisheries University to units in Ningbo and Tianjin cities and Guangdong province in 1958, 1959, and 1961. Thus, a number of isolates from Southern China were similar to the North American bullfrog isolates from Japan, which may be explained by the long-term trade exchange.

Amphibian commerce is a possible source of pathogen pollution (Picco and Collins, 2008). The unregulated trade and movement of animals infected with ranaviruses will definitely result in the potential exposure of native species and populations to virulent pathogens (Cunningham *et al.*, 2003). International cooperation is therefore required to determine potential sources of ranaviral infections and implement effective management strategies.

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Conflict of interest statement

We do not have any conflict of interest.

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