Development of a reverse transcription polymerase chain reaction for the detection of severe fever with thrombocytopenia syndrome virus from suspected infected animals

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Abstract

Background

Severe fever with thrombocytopenia syndrome virus (SFTSV) causes severe hemorrhagic fever in humans and cats. Clinical symptoms of SFTS-infected cats resemble to those of SFTS patients and SFTS-contracted cats shows high levels of viral RNA loads in the serum and body fluids. Due to the risk of direct infection from SFTS-infected cats to human, it is important to diagnose SFTS-suspected animals.

Methodology/Principle findings

Four primer sets were newly designed from consensus sequences constructed by 108 strains of SFTSV. A reverse transcription polymerase chain reaction (RT-PCR) with these four primer sets were successfully and specifically detected several clades of SFTSV. Their limits of detection are 1-10 copies/reaction. By this RT-PCR, 5 cat cases among 56 SFTS-suspected animal cases were diagnosed as SFTS. From these cats, IgM or IgG against SFTSV were detected by enzyme-linked immunosorbent assay (ELISA), but not neutralizing antibodies by plaque reduction neutralization titer (PRNT) test. This phenomenon is similar to those of fatal SFTS patients.

Conclusion/Significance

This newly developed RT-PCR could detect SFTSV RNA of several clades from SFTS-suspected animals. In addition to ELISA and PRNT test, the useful laboratory diagnosis systems of SFTS-suspected animals has been made in this study.

Author summary

This study developed RT-PCR to detect SFTS animal cases. This assay could detect SFTSV RNA belonging to different clades. Cats diagnosed as SFTS had IgM or IgG, but not neutralizing antibodies. SFTS cat cases were distributed in the area where SFTS patients have been reported highly, indicating the establishment of the circulation of SFTSV in the environment. These diagnostic assays could be helpful tools to detect and not to miss SFTS animal cases.

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging viral hemorrhagic fever that was first identified in China (1), with cases since reported in Japan, South Korea, Vietnam and Taiwan (2, 3). SFTS virus (SFTSV) has been identified as a causative virus of SFTS belonging to the genus *Banyangvirus*, family *Phenuiviridae*, order *Bunyavirales*.

SFTS has been considered to be mainly transmitted by tick bites. Ticks infest a variety of animals, and viral RNA and antibodies against SFTSV have been detected in wild animals, domestic animals and companion animals, such as dogs and cats [1-5]. Since these animals show no clinical symptoms, they have been considered subclinically infected with SFTSV. In recent years, SFTS patients without a history of tick bites have been reported, and they are considered to have received the virus through transmission from animals, such as cats and dogs [4, 6]. Furthermore, it has been shown that cheetahs [7], cats [8] and dogs can contract SFTS (*manuscript in preparation*). Thus, it is important to diagnose SFTS-suspected animals. Seven to eight clades of SFTSV are reportedly spread throughout Japan, China and South Korea [9].

In this study, a reverse transcription polymerase chain reaction (RT-PCR) was developed to establish a laboratory diagnosis system for detecting all the clades of SFTSV in the specimens of SFTS-suspected animals.

Materials and Methods

Serum samples

The samples, including serum and oral and rectal swabs, were collected from SFTS-suspected cats and dogs at veterinary hospitals throughout Japan from August 2017 to March 2019. Cats exhibiting a fever (>39 °C), leukocytopenia (<2000 /µL), thrombocytopenia (<200,000 /µL) and elevated levels of AST, ALT and CK were suspected of having SFTS. Clinical information was provided by veterinarians.

A phylogenic analysis for primer design

The nucleotide sequences of 100 strains of SFTSV S segment and M segment were selected randomly from Genbank to cover all the clades and aligned and phylogenetically analyzed (Figure 1). In brief, phylogenic trees were constructed using with the maximum likelihood method with the Tamura-Nei model using the MEGA 7 software program [10]. The robustness of the resulting branching patterns was tested using the bootstrap method with 1,000 replicates. From this analysis, it was confirmed that the seven to eight clades of SFTSV correlate with their geographical location, as has been reported previously [9]. The nucleotide identity, determined using the Bioedit sequence alignment editor [11], was 94.1%-99.1% in S segment, and 93%-99.7% in M segment among clusters (Figure 2). The consensus sequences of S segment and M segment among the different strains were selected using the Bioedit program, and primers were designed by the NCBI Primer-BLAST [12] from the consensus sequences.

RT-PCR

RNAs isolated from the culture supernatants of three strains of SFTSV belonging to different clades - SPL010 (J1 clade, accession No. AB817999), cat#1 (C4 clade, accession No. DRA007207) and HB29 (C3 clade, accession No. NC_018137) - were used as positive controls. RNA was extracted using a High Pure Viral RNA Kit (Roche, Mannheim, Germany) as previously reported study (Park ES). RT-PCR was performed using the Superscript III one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) under the following conditions: RT at 55 °C for 30 min, inactivation at 95 °C for 2 min, and then 40 cycles of PCR at 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s, followed by extension at 68 °C for 5 min. Four primer pairs successfully detected the RNAs of the three different SFTSV strains with a detection limit of 1-10 copies/reaction (Figures 3 and 4 and Table 1).

SFTSV detection from samples using four designated primers

Total RNAs were extracted from the specimens of dogs and cats using ISOGEN (Wako, Osaka, Japan) and a precipitation carrier (Ethachinmate; Wako), according to the manufacturer's instructions. RT-PCR was performed using a Superscript III one-step RT-PCR system with platinum Taq DNA polymerase (Invitrogen) with four sets of specific primers. Samples with more than two positive bands were considered SFTSV RNA-positive.

Amplification of viral genome of S segment and phylogenetic analysis

RT-PCR-positive RNAs were used to determine the viral genome of the S segment for a phylogenetic analysis. RT-PCR was performed using a Superscript III one-step RT-PCR system with platinum Taq DNA polymerase (Invitrogen) with primers covering the entire S segment region according to a previously reported study [9]. The PCR products were determined by electrophoresis on 1% agarose gels with GR Red Loading Buffer (GRR-1000, Bio-Craft, Tokyo, Japan). The PCR products were then extracted and purified using an illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK). The samples were sequenced using the general Sanger sequencing technique.

The nucleotide sequences determined in this study were deposited in the DDBJ GenBank databases. For the phylogenetic analysis, three nucleotide data points per cluster were selected. The sequence alignment was computed using the Clustal W program of MEGA 7 software program. The phylogenetic tree was constructed using the maximum likelihood method based on the Tamura-Nei model of the MEGA program. The confidence of the tree was tested using 1000 bootstrap replications.

Detection of IgM and IgG in cats by an enzyme-linked immunosorbent assay (ELISA)

Antibodies against SFTSV were detected by an ELISA, essentially performed as in the previously described study [13]. In brief, SFTSV- or mock-infected Huh7 cells were lysed in 1% NP40 in phosphate-buffered saline (PBS), ultraviolet (UV)-irradiated to completely inactivate SFTSV, and then clarified by centrifugation at 12,000 rpm for 10 min. The lysates were coated onto the ELISA plate (Nunc-Immuno^{TM} plate; Thermo Fisher Scientific, Roskilde, Denmark). The antigen-coated wells were then blocked with 20% Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) in PBS (blocking solution) at room temperature for 1 h. Sera of cats and dogs were inactivated at 56 °C for 30 min and serially 4-fold diluted from 1:100 to 1:6400 in the blocking solution at 37 °C for 1 h. Horseradish (HRD)-conjugated goat anti-feline IgG Fc and HRD-conjugated goat anti-feline IgM (Novus biologicals), and HRD-conjugated goat anti-dog IgM(μ) and HRD-conjugated sheep anti-dog IgG(H) were used to detect IgM and IgG antibodies in cats and dogs, respectively. The reaction was finally visualized by a substrate for HRP (ABST, 2, 2azinobis (3-ethylbenzthiazolinesulfonic acid); Roche, Mannheim, Germany) for 30 min at room temperature. The optical density (OD) at 405 nm was measured with an iMark^{TM} microplate reader (Bio-Rad, Tokyo, Japan). The OD values in the mock-antigen coated well were subtracted from the OD value in the respective SFTSV-antigen coated wells. The cut-off OD value was set as the average subtracted OD value plus three times the standard deviation (SD), that is, mean + 3SD, of SFTS-negative serum that had been confirmed by an indirect immunofluorescent antibody assay using SFTSV-infected Vero cells. The sera were considered positive when the OD values were above the cut-off value. RT-PCR was performed to detect SFTSV in clinical animal specimens.

The 50% plaque reduction neutralization titer (PRNT$_{50}$)

The PRNT test was performed to determine the neutralizing antibodies against SFTSV using Vero cells (ATCC), according to previously reported studies. Approximately 100 plaque-forming units of the HB29 strain of SFTSV were mixed with serially diluted heat-inactivated sera and incubated for 1 h at 37 °C and then inoculated into confluent monolayers of Vero cell in 12-well plates for 1 h at 37 °C. The inocula were removed, and the cells were washed once with DMEM containing 2% FBS and kanamycin and then cultured at 37 °C in a 5% CO_2 incubator with DMEM containing 2% FBS, Kanamycin and 1% methylcellulose for 1 week. Cultured cells were fixed with 10% buffered formalin and exposed to UV radiation to inactivate the virus. The cells were permeabilized with 0.1% Triton X-100, followed by incubation with rabbit antibodies against SFTSV-N as primary antibodies and HRP-conjugated recombinant protein A/G (Cat. No. 32490, Thermo Scientific, Rockford, IL, USA) as secondary antibodies. Plaques were visualized with 3, 3'-diaminobenzidine tetrahydrochloride (Peroxidase stain DAB kit [Brown stain]; Nacalai

Tesque). The PRNT50 value was determined as the reciprocal of the highest dilution at which the number of the plaques was below 50% of the number calculated without cat serum.

Results

Four primer pairs (2 for the S segment and 2 for the M segment) successfully detected the RNAs of the three SFTSV strains belonging to different clusters with a detection limit of 1-10 copies/reaction (Figures 3 and 4 and Table 1). For the detection of SFTSV RNA from samples, these four primer sets were used.

From August 2017 to March 2019, 56 cases were collected, and RT-PCR was performed to detect SFTSV RNA. Among them, SFTSV RNA was detected in the sera of five cats. The PCR products were confirmed with RT-PCR using all four sets of primer pairs (Figure 5). The positive samples were evaluated to determine the nucleotide of the S segment (Table 2). These nucleotide sequences of the S segment from five cases were phylogenetically analyzed with the corresponding segment of the Heartland virus as an outgroup (Figure 6). As a result, four strains were clustered into genotype J1, and one strain was clustered into genotype J3 of the Japanese clade, according to previous studies.

In addition to SFTSV RNA, antibodies against SFTSV were detected in the sera of the five cats that were positive on RT-PCR (Table 3). Three samples had IgM and IgG against SFTSV, and two had IgM or IgG, respectively (Table 3). Serum samples were collected at a one-week interval from one case. IgM was detected in these two interval sera, and IgG was detected in the serum collected one week later. This seroconversion pattern was similar to that of our previous study. Antibodies were not detected in the RT-PCR-negative animals.

The neutralizing antibodies against SFTSV were then measured with the PRNT_{50} according to our previous study. The titer of neutralizing antibodies was below the limit of detection, indicating that the antibodies detected by the ELISA were not functional, similar to those of fatal human cases.

Discussion

In this study, four primer sets were able to detect SFTSV RNA belonging to different genotypes with a low detection limit. Two pairs were specific for the S segment, and two pairs were specific for the M segment. SFTSV RNAs were detected from five cases using these primers. Four positive bands were observed in all five cases. These positive cases were distributed in the same region where human SFTS cases have been reported (Table 4). The sites at which two cases were detected were close to each other. The genotype of these strains was J1, showing 99.3%-99.7% homology (data not shown). These findings are believed to establish the hot spot and circulation of SFTSV among ticks and animals. The ages of the cats ranged from 9 months to 15 years old. The period of disease onset was from January to October. All of these cats were kept both indoors and outdoors. In addition, four cases had a tick-bite history, indicating the transmission of SFTSV by tick. Their clinical symptoms were similar to those described in previous reports. Thus, these cats were diagnosed with SFTS.

Five cats had IgM and/or IgG against SFTSV, determined by an ELISA. However, the titer of neutralizing antibodies was below the limit of detection. The serum specimens of the cats were collected within one week after the onset. Given that the level of neutralizing antibodies in the surviving cats was elevated in our previous study [8], the insufficient induction or non-function of neutralizing antibodies might result in the severe onset in cats. Given that B cell lineages, such as plasmablasts, can be the target of SFTSV, this may be a plausible result.

In conclusion, the RT-PCR approach developed in the present study and the IgM- and IgG-ELISA performed to detect SFTS-specific antibodies were useful for making a laboratory diagnosis of SFTS-suspected cats and dogs.

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Figure legends

Figure 1. Phylogenic trees of the SFTSV genome for the S (A) and M (B) segments. Strains that were identified in China, Japan and Korea are indicated by red, blue and red, respectively.

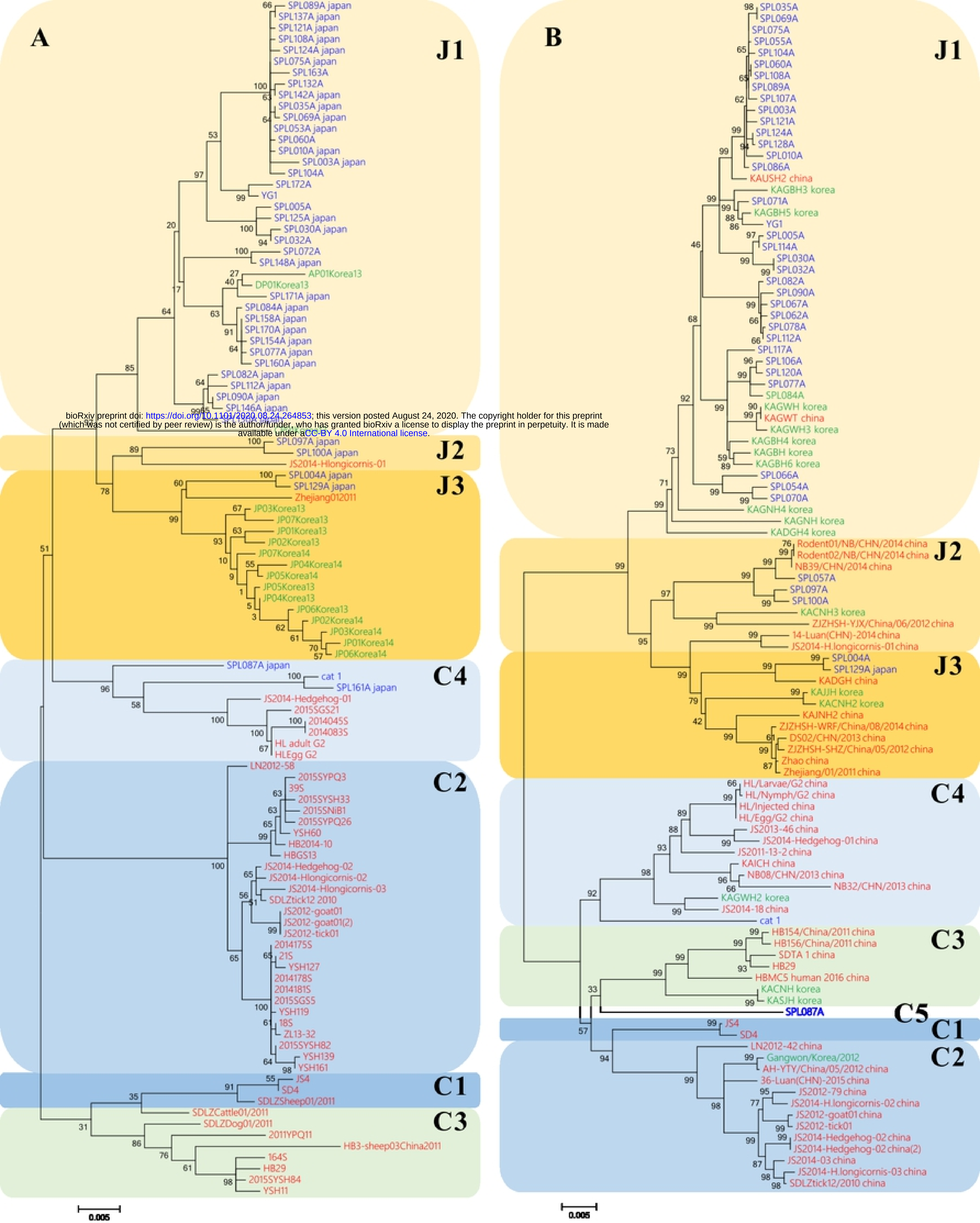
Figure 2. The nucleotide identities among SFTSV clades of S (A) and M (B) segments. Two strains of each clade were selected randomly, and the nucleotide identities were determined using the Bioedit software program. (A) S segment; J1 (Japan 1: SPL010A, Korea 1: CP01Korea13), J2 (Japan 2: SPL097A, China 1: JS2014-Hlongicornis-01), J3 (Japan 3: SPL129A, Korea 2: JP03Korea14), C1 (China 2: JS2, China 3: SDLZSheep01/2011), C2 (China 4: 2015SYSH33, China 5: 2014181S), C3 (China 6: SDLZDog01/2011, China 7: HB29) and C4 (Japan 4: cat#1, China 8: HLEgg_G2). M segment; J1 (Japan 1: SPL010A, Korea 1: KAGWH_korea), J2 (Japan 2: SPL057A, Japan 3: SPL100A), J3 (Japan 4: SPL004A, Korea 2: KAJJH_korea), C1 (China 1: JS4, China 2: SD4), C2 (Korea 3: Gangwon/korea/2012, China 3: SDLZtick12/2010_china), C3 (China 4: HB154/China/2011_china, China 5: HB29) and C4 (China 6: HL/Nymph/G2_china, Japan 5: cat#1).

Figure 3. RT-PCR using primer 2 (S segment) electrophoresis of the SPL010 strain for the confirmation of the detection limit. Viral RNA was extracted from the SPL010 strain. 10^3-10^0 copies/reaction and mock sample (from left to right). A PCR product of 125 bp was observed.

Figure 4. RT-PCR using primer 4 (M segment) electrophoresis of HB29 strain for the confirmation of the detection limit. HB29-M/pKS336 plasmid was used. 10^8-10^0 copies/reaction and mock sample (from left to right). A PCR product of 179 bp was observed.

Figure 5. RT-PCR electrophoresis of an SFTS case (Case 1). The PCR product bands were observed. I: sample RNA, II: positive control, III: negative control.

Figure 6. Phylogenic trees of detected SFTSV genome in this study (red) and reference SFTSV genome (black) for the S segments.


A
J1
J2
J3
C4
C2
C1
C3
SPL089A japan
SPL137A japan
SPL121A japan
SPL108A japan
SPL124A japan
SPL075A japan
SPL163A
SPL132A
SPL142A japan
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SPL069A japan
SPL053A japan
SPL060A
SPL010A japan
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SPL104A
SPL172A
YG1
SPL005A
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SPL030A japan
SPL032A
SPL072A
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AP01Korea13
DP01Korea13
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2015SYPQ26
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HBGS13
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JS2014-Hlongicornis-03
SDLZtick12 2010
JS2012-goat01
JS2012-goat01(2)
JS2012-tick01
2014175S
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YSH127
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2014181S
2015SGS5
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YSH161
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B
J1
J2
J3
C4
C3
C5
C1
C2
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JS2014-H.longicornis-01 china
SPL004A
SPL129A japan
KADGH china
KAJJH korea
KACNH2 korea
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DS02/CHN/2013 china
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NB32/CHN/2013 china
KAGWH2 korea
JS2014-18 china
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HB154/China/2011 china
HB156/China/2011 china
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HB29
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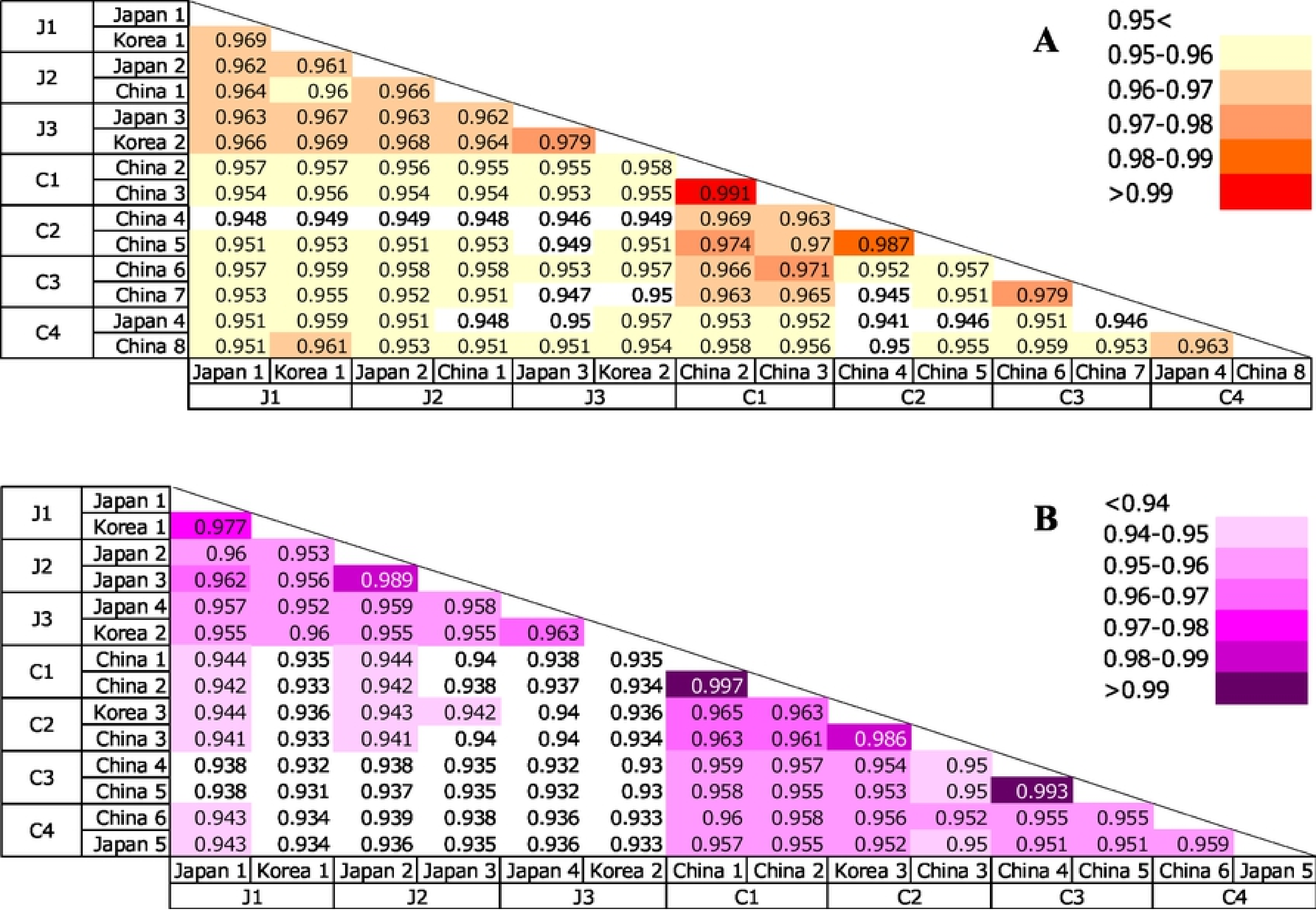
Figure 1



A

0.95<
0.95-0.96
0.96-0.97
0.97-0.98
0.98-0.99
>0.99

J1	Japan 1													
	Korea 1	0.969												
J2	Japan 2	0.962	0.961											
	China 1	0.964	0.96	0.966										
J3	Japan 3	0.963	0.967	0.963	0.962									
	Korea 2	0.966	0.969	0.968	0.964	0.979								
C1	China 2	0.957	0.957	0.956	0.955	0.955	0.958							
	China 3	0.954	0.956	0.954	0.954	0.953	0.955	0.991						
C2	China 4	0.948	0.949	0.949	0.948	0.946	0.949	0.969	0.963					
	China 5	0.951	0.953	0.951	0.953	0.949	0.951	0.974	0.97	0.987				
C3	China 6	0.957	0.959	0.958	0.958	0.953	0.957	0.966	0.971	0.952	0.957			
	China 7	0.953	0.955	0.952	0.951	0.947	0.95	0.963	0.965	0.945	0.951	0.979		
C4	Japan 4	0.951	0.959	0.951	0.948	0.95	0.957	0.953	0.952	0.941	0.946	0.951	0.946	
	China 8	0.951	0.961	0.953	0.951	0.951	0.954	0.958	0.956	0.95	0.955	0.959	0.953	0.963
		Japan 1	Korea 1	Japan 2	China 1	Japan 3	Korea 2	China 2	China 3	China 4	China 5	China 6	China 7	Japan 4	China 8
		J1		J2		J3		C1		C2		C3		C4	

B

<0.94
0.94-0.95
0.95-0.96
0.96-0.97
0.97-0.98
0.98-0.99
>0.99

J1	Japan 1													
	Korea 1	0.977												
J2	Japan 2	0.96	0.953											
	Japan 3	0.962	0.956	0.989										
J3	Japan 4	0.957	0.952	0.959	0.958									
	Korea 2	0.955	0.96	0.955	0.955	0.963								
C1	China 1	0.944	0.935	0.944	0.94	0.938	0.935							
	China 2	0.942	0.933	0.942	0.938	0.937	0.934	0.997						
C2	Korea 3	0.944	0.936	0.943	0.942	0.94	0.936	0.965	0.963					
	China 3	0.941	0.933	0.941	0.94	0.94	0.934	0.963	0.961	0.986				
C3	China 4	0.938	0.932	0.938	0.935	0.932	0.93	0.959	0.957	0.954	0.95			
	China 5	0.938	0.931	0.937	0.935	0.932	0.93	0.958	0.955	0.953	0.95	0.993		
C4	China 6	0.943	0.934	0.939	0.938	0.936	0.933	0.96	0.958	0.956	0.952	0.955	0.955	
	Japan 5	0.943	0.934	0.936	0.935	0.936	0.933	0.957	0.955	0.952	0.95	0.951	0.951	0.959
		Japan 1	Korea 1	Japan 2	Japan 3	Japan 4	Korea 2	China 1	China 2	Korea 3	China 3	China 4	China 5	China 6	Japan 5
		J1		J2		J3		C1		C2		C3		C4	

Figure 2

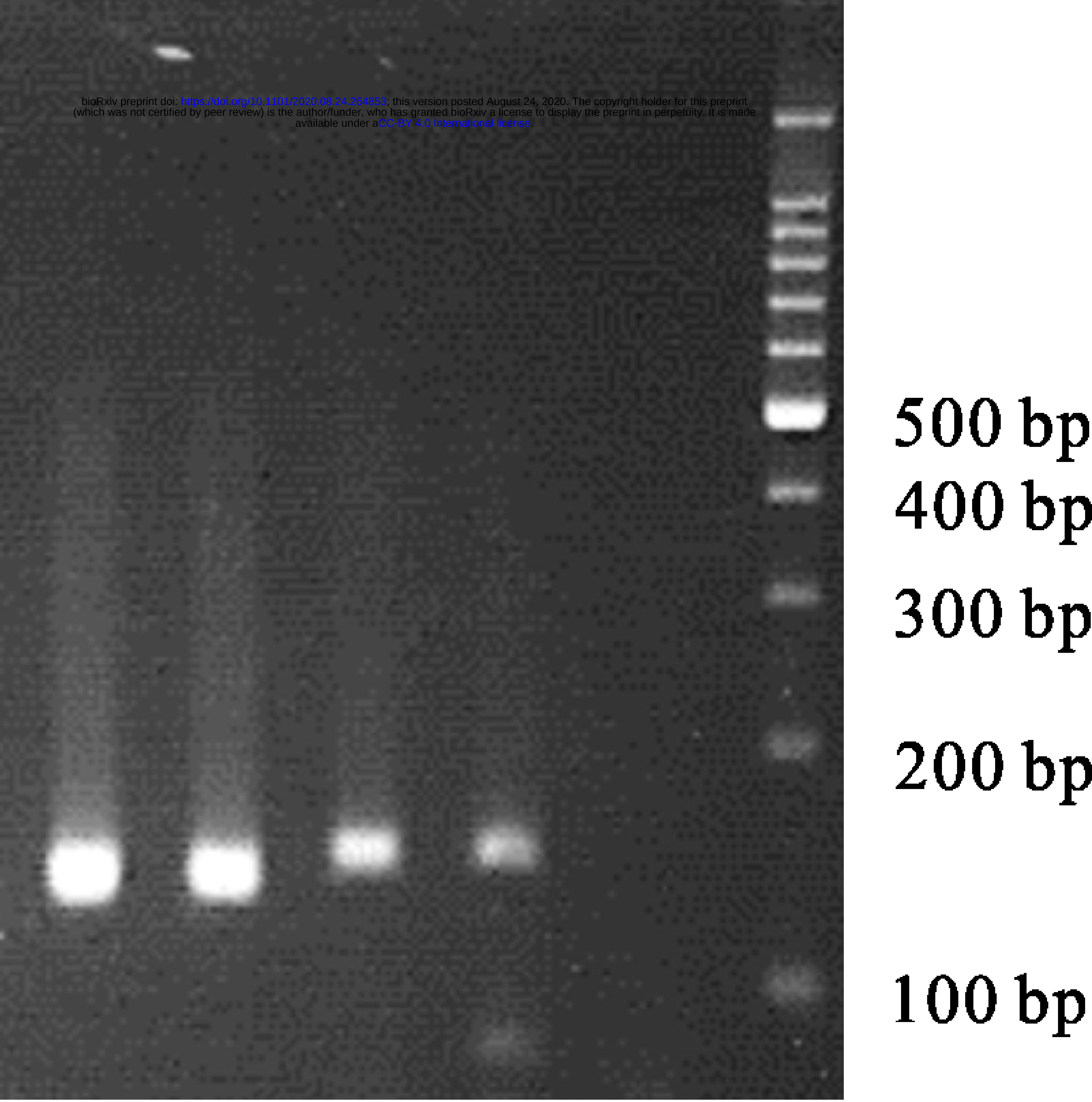


Figure 3

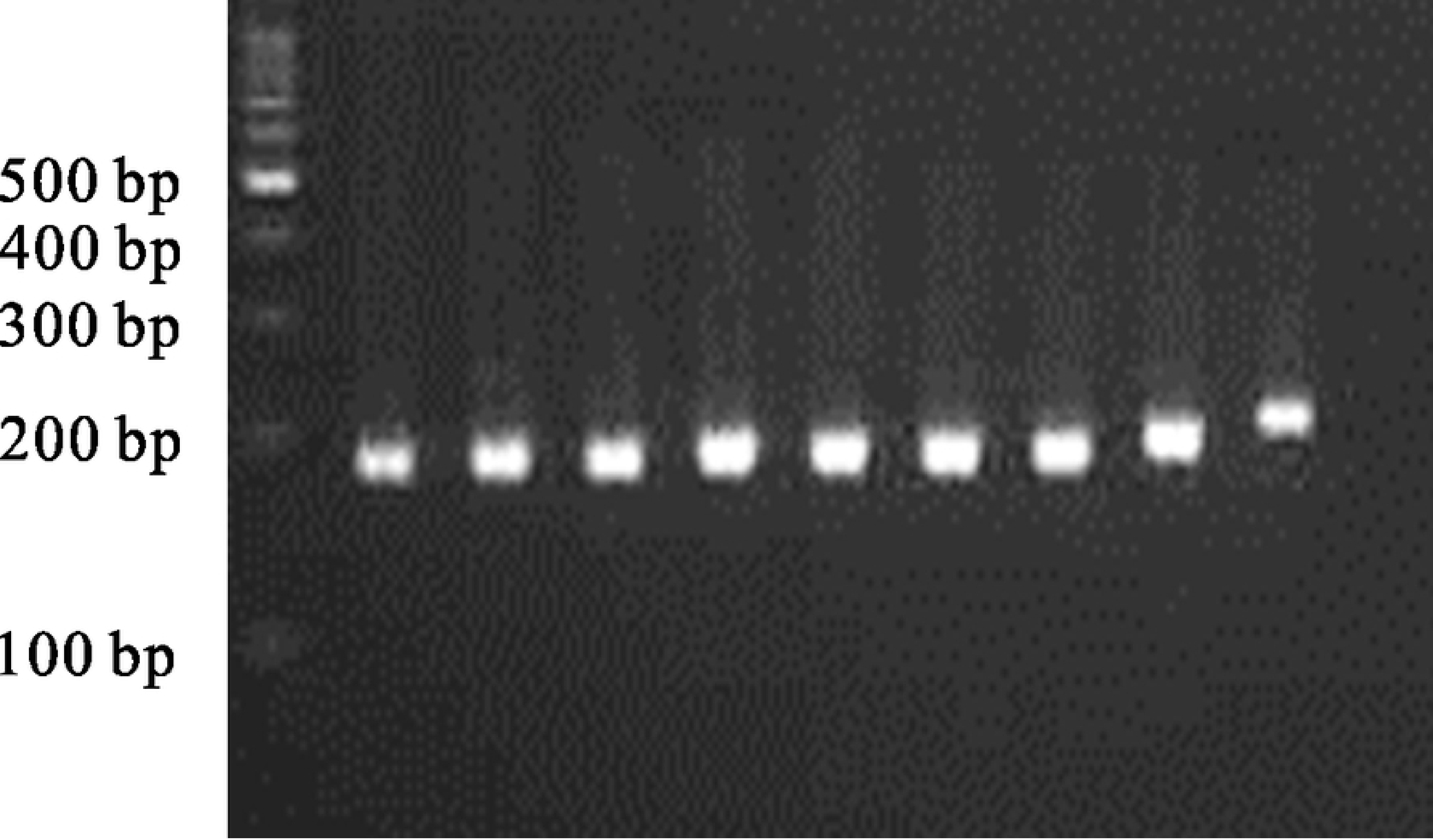


Figure 4

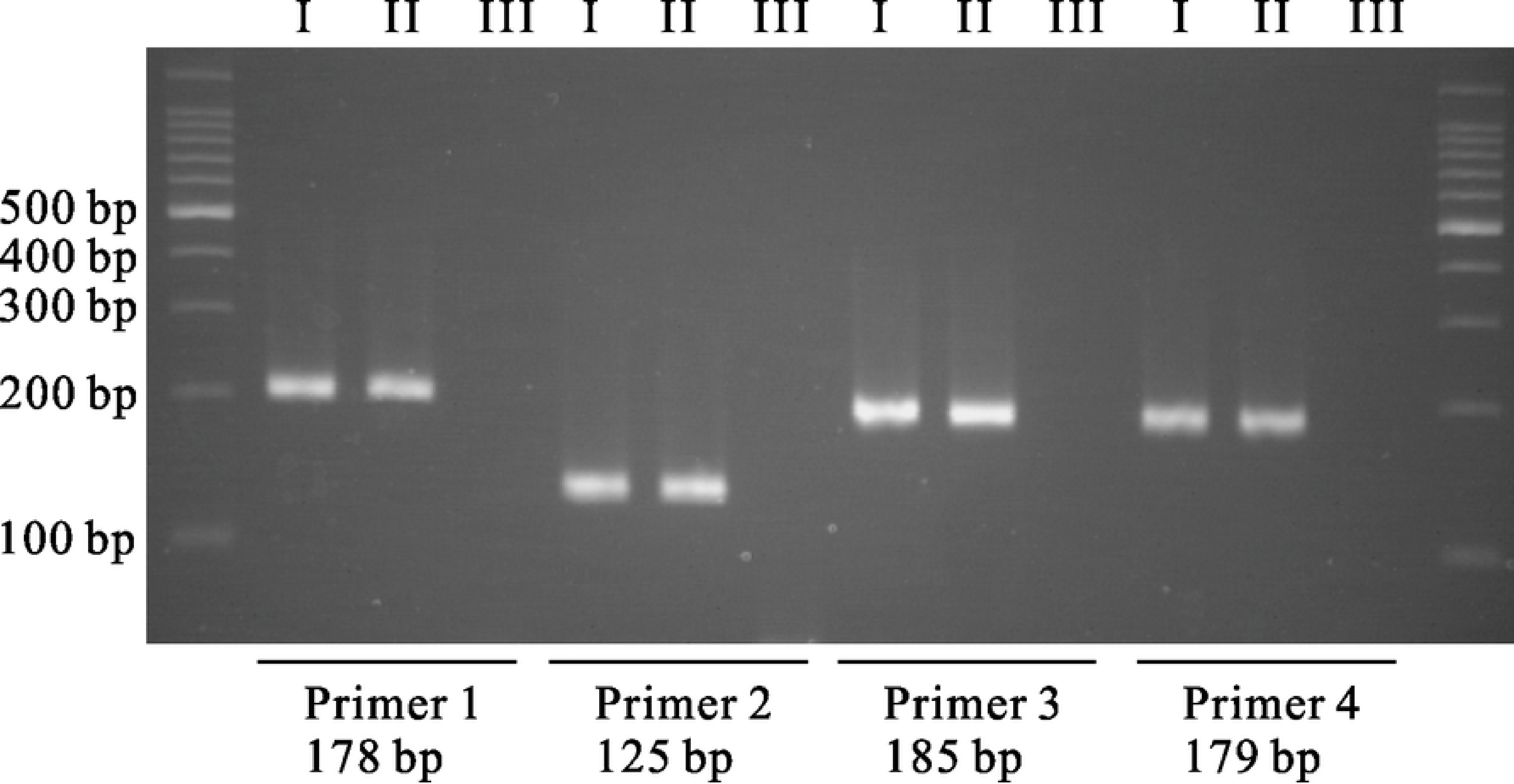

I
II
III
I
II
III
I
II
III
I
II
III
500 bp
400 bp
300 bp
200 bp
100 bp
Primer 1
178 bp
Primer 2
125 bp
Primer 3
185 bp
Primer 4
179 bp

Figure 5

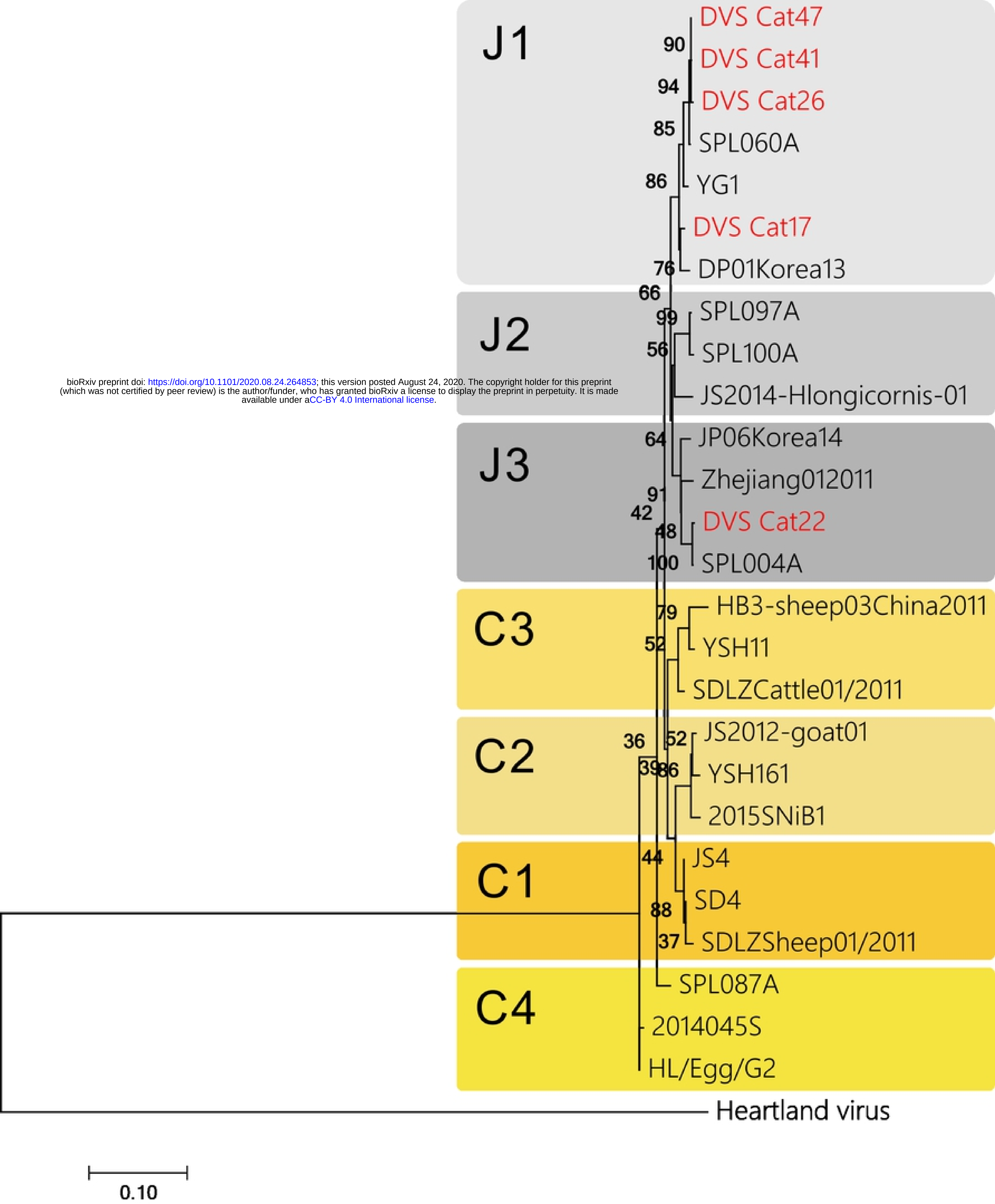

J1
DVS Cat47
90
DVS Cat41
94
DVS Cat26
85
SPL060A
86
YG1
DVS Cat17
76
DP01Korea13
66
J2
99
SPL097A
56
SPL100A
JS2014-Hlongicornis-01
J3
64
JP06Korea14
Zhejiang012011
91
42
DVS Cat22
48
100
SPL004A
C3
79
HB3-sheep03China2011
52
YSH11
SDLZCattle01/2011
C2
36
52
JS2012-goat01
39
86
YSH161
2015SNiB1
C1
44
JS4
88
SD4
37
SDLZSheep01/2011
C4
SPL087A
2014045S
HL/Egg/G2
Heartland virus
0.10

Figure 6

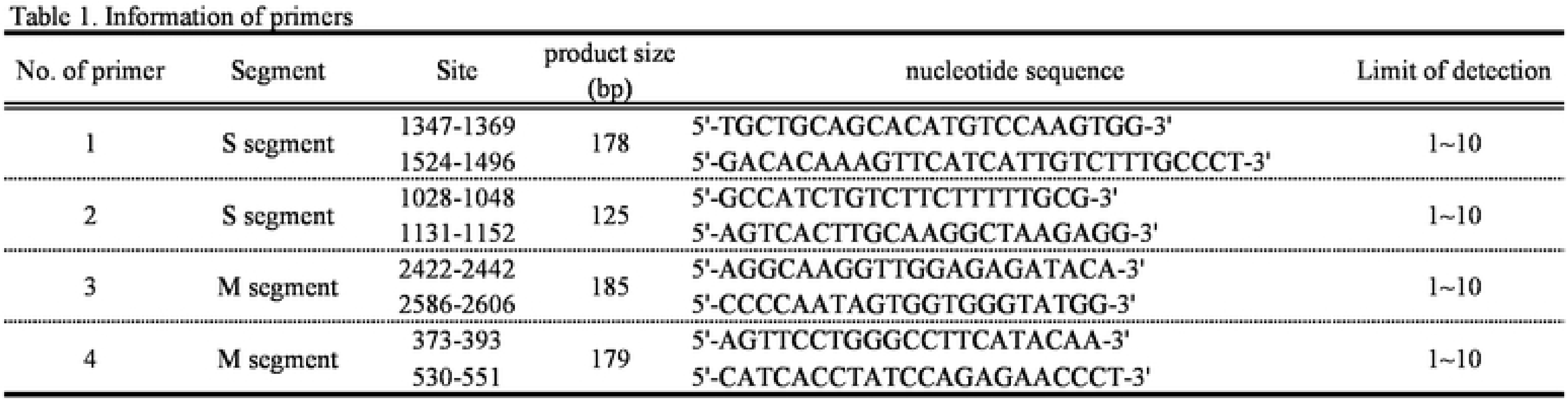


Table 1. Information of primers

No. of primer	Segment	Site	product size (bp)	nucleotide sequence	Limit of detection
1	S segment	1347-1369 1524-1496	178	5'-TGCTGCAGCACATGTCCAAGTGG-3' 5'-GACACAAAGTTCATCATTGTCTTTGCCCT-3'	1~10
2	S segment	1028-1048 1131-1152	125	5'-GCCATCTGTCTTCTTTTTGCG-3' 5'-AGTCACTTGCAAGGCTAAGAGG-3'	1~10
3	M segment	2422-2442 2586-2606	185	5'-AGGCAAGGTTGGAGAGATACA-3' 5'-CCCCAATAGTGGTGGGTATGG-3'	1~10
4	M segment	373-393 530-551	179	5'-AGTTCCTGGGCCTTCATACAA-3' 5'-CATCACCTATCCAGAGAACCCT-3'	1~10

Table 1

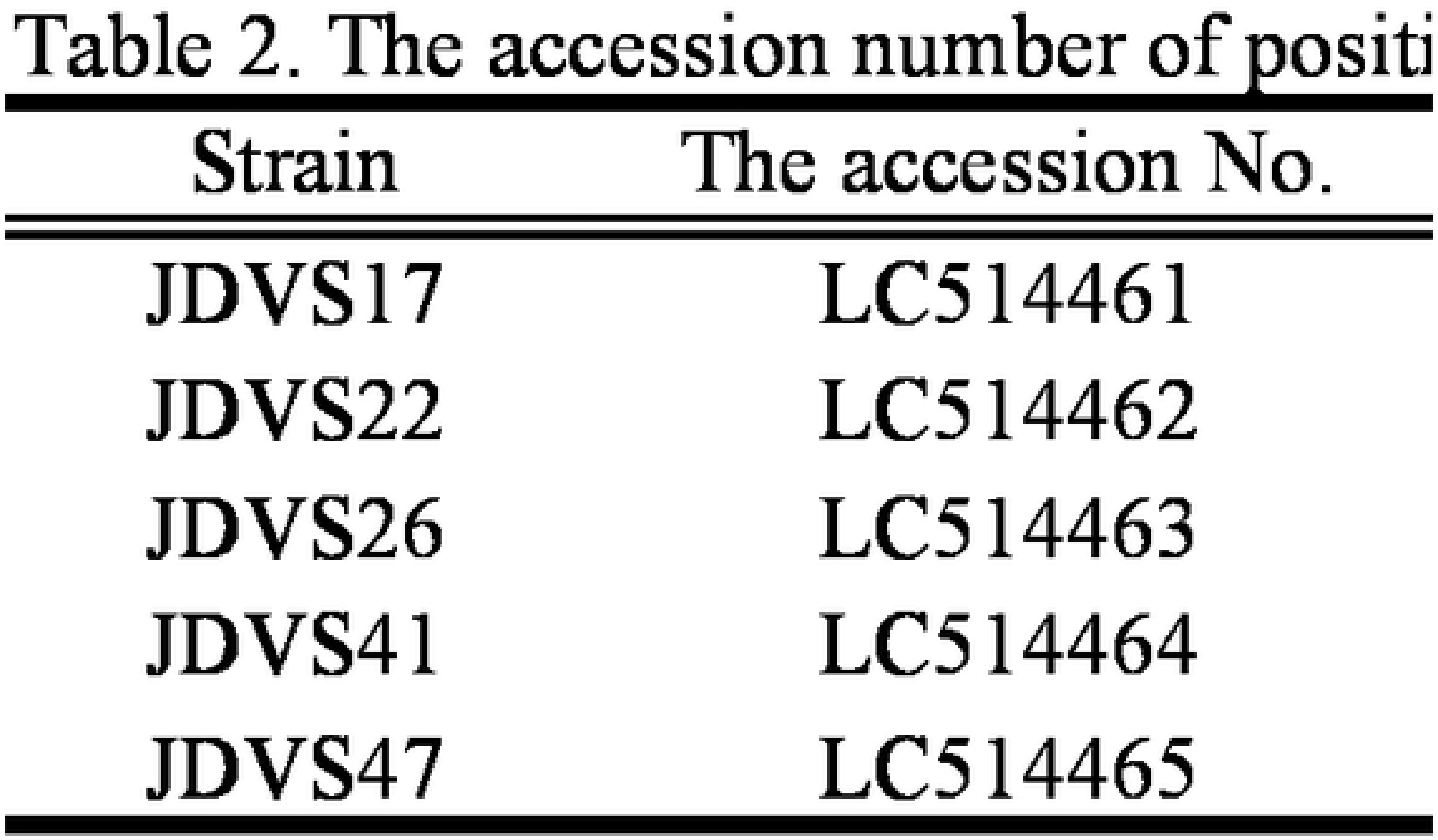


Table 2. The accession number of positi

Strain	The accession No.
JDVS17	LC514461
JDVS22	LC514462
JDVS26	LC514463
JDVS41	LC514464
JDVS47	LC514465

Table 2

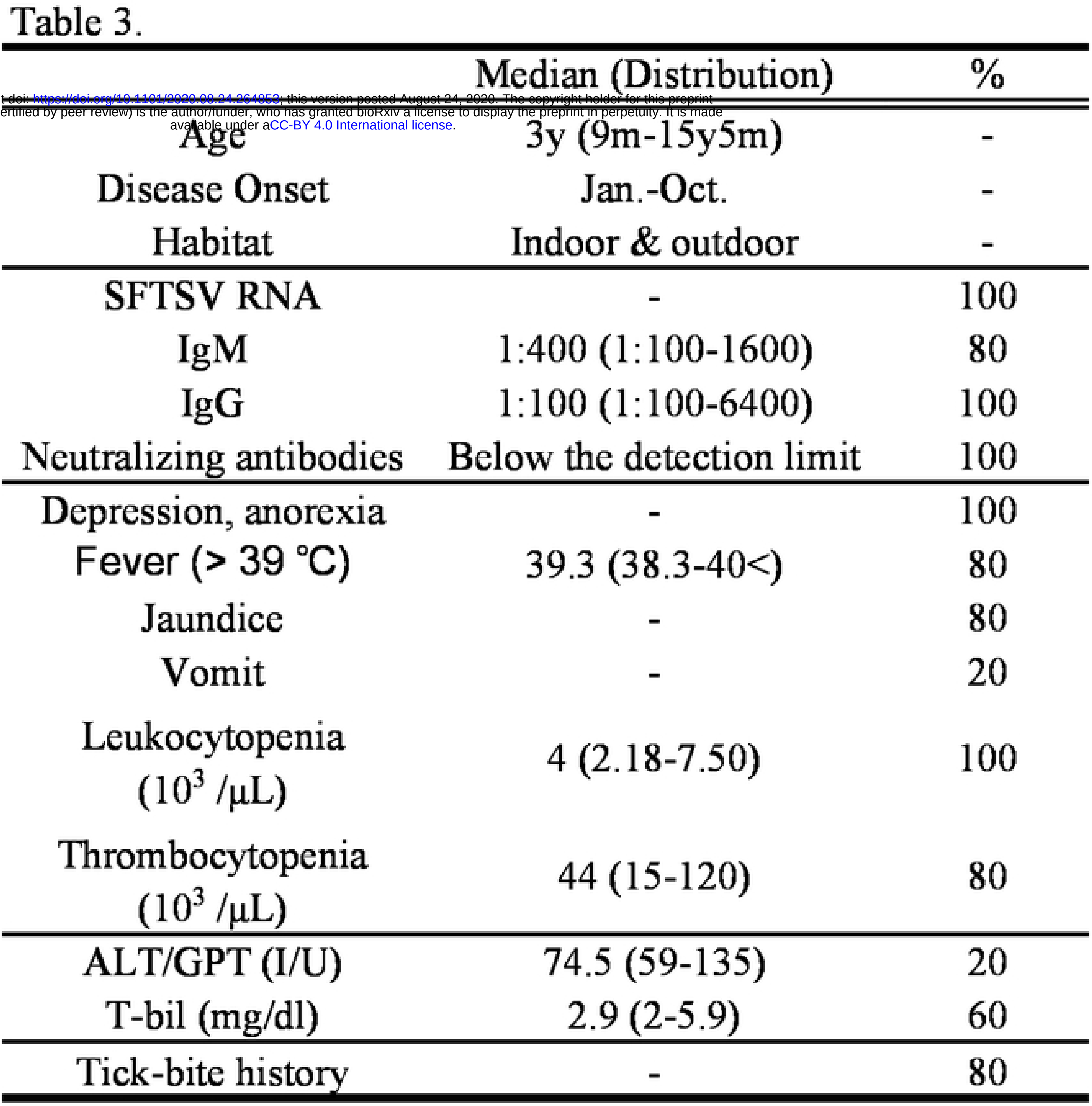


Table 3.

	Median (Distribution)	%
Age	3y (9m-15y5m)	-
Disease Onset	Jan.-Oct.	-
Habitat	Indoor & outdoor	-
SFTSV RNA	-	100
IgM	1:400 (1:100-1600)	80
IgG	1:100 (1:100-6400)	100
Neutralizing antibodies	Below the detection limit	100
Depression, anorexia	-	100
Fever (> 39 ℃)	39.3 (38.3-40<)	80
Jaundice	-	80
Vomit	-	20
Leukocytopenia (10^3 /μL)	4 (2.18-7.50)	100
Thrombocytopenia (10^3 /μL)	44 (15-120)	80
ALT/GPT (I/U)	74.5 (59-135)	20
T-bil (mg/dl)	2.9 (2-5.9)	60
Tick-bite history	-	80

Table 3

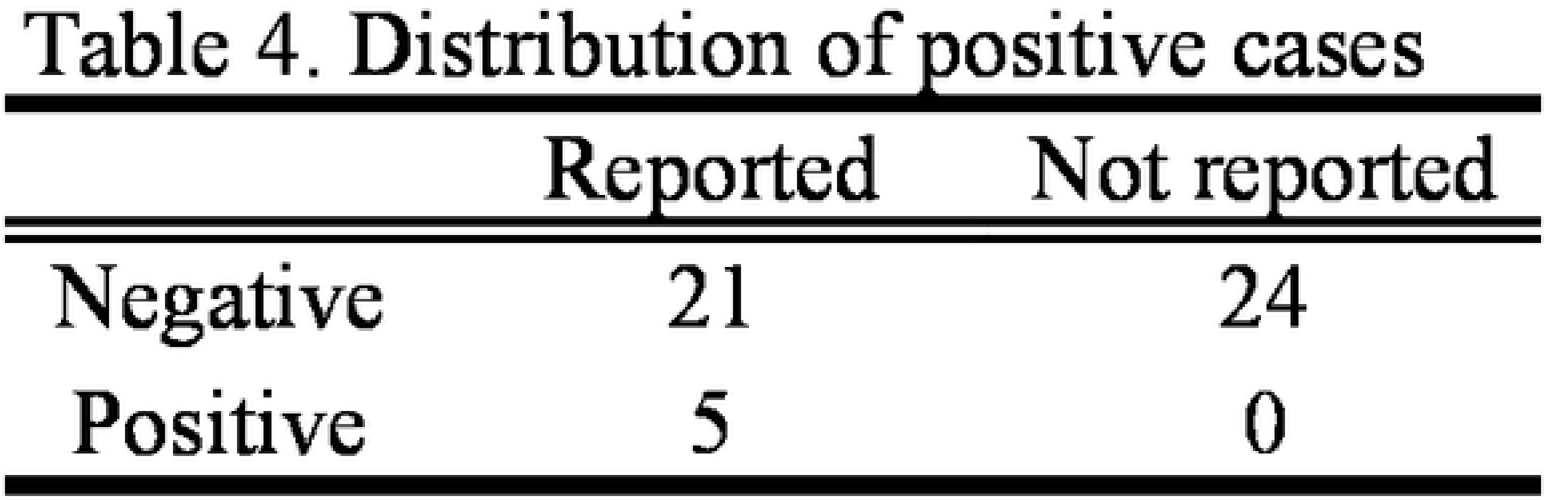


Table 4. Distribution of positive cases

	Reported	Not reported
Negative	21	24
Positive	5	0

Table 4