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Detection of novel ferret coronaviruses and evidence of recombination among ferret coronaviruses

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Abstract In an epidemiological study of ferret coronaviruses (FRCoVs), novel FRCoV strains (Saitama-1 and Aichi-1) were detected by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequence analysis of partial RNA-dependent RNA polymerase (RdRp) genes. Phylogenetic analysis indicated that these strains belonged to different clusters from other FRCoV strains. Next, the nucleotide sequence of the 3′-terminal region of Saitama-1 (8271 bases) strain was determined and compared with those of the other FRCoVs, indicating that the Saitama-1 strain differed from the previously reported MSU-1 and MSU-2 strains in the regions encoding spike (S) protein, nucleocapsid, and open reading frame 7b. Furthermore, the results of SimPlot analysis indicated that FRCoV (MSU-2 strain) emerged via a recombination event of S protein between the MSU-1 and Saitama-1 strains. This mechanism is similar to that responsible for the emergence of type II feline coronavirus. This information will be useful for understanding the pathogenesis of FRCoV in ferrets.

Keywords Ferret coronavirus (FRCoV) · Novel genotype · Recombination

Epizootic catarrhal enteritis (ECE) in ferrets (*Mustelo putorius furo*) was first reported in the United States in the early 1990s as a new enteric disease [16]. A novel alphacoronavirus, ferret coronavirus (FRCoV), was detected as the causative agent of ECE in 2000, and was designated as ferret enteric coronavirus (FRECV) [16, 17]. General clinical signs of ECE include vomiting, lethargy, anorexia, and foul-smelling, green mucous-laden diarrhea [17]. FRCoV was also reported as the causative agent of a feline infectious peritonitis (FIP)-like disease in 2006. This FRCoV was designated as ferret systemic coronavirus

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(FRSCV) [2, 3, 6]. The characteristic clinical sign of FIP-like disease is large palpable intra-abdominal masses, like dry-type FIP [2, 3, 6]. In addition, there are two genotypes of FRCoV based on differences in the spike (S) gene. This suggests that genotype I is associated with FIP-like disease and genotype II is associated with ECE [18]. However, we previously showed that there was no significant relationship between the genotypes of FRCoV and disease in Japan [14]. In addition, genotype I FRCoV was also detected in numerous asymptomatic ferrets in the Netherlands [9]. The relationship between FRCoV genotype and clinical symptoms thus remains unclear.

Reverse transcription-polymerase chain reaction (RT-PCR) was used for epidemiological study of FRCoV infection. FRCoV gene detection in 56–61 % of ferrets in Japan and the Netherlands has been reported [9, 14]. Recently, an enzyme-linked immunosorbent assay (ELISA) using recombinant nucleocapsid (N) protein of FRCoV had been established, indicating that many ferrets possess antibodies against FRCoV [7]. However, FRCoV has not yet been isolated.

In animal hospitals throughout Japan, 201 fecal samples were collected from domestic ferrets between Aug 1, 2012 and Dec 8, 2015 and were examined by RT-PCR. RNA was extracted from fecal samples using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and RT-PCR was performed using a QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). For specific and sensitive detection of FRCoV, FRCoV-specific primers, FRCoV RdRp-F1 (5′-GTT GGT TGC TGC ACA CAT AG-3′) and FRCoV RdRp-R1 (5′-GGA GAA GTG CTT ACG CAA ATA-3′) were used [14]. Coronavirus (CoV)-consensus primers, IN-6 (5′-GGT TGG GAC TAT CCT AAG TGT GA-3′) and IN-7 (5′-CCA TCA TCA GAT AGA ATC ATC ATA-3′), were also used for the detection of coronaviruses [8]. Both primer pairs target partial RNA-dependent RNA polymerase (RdRp) gene. The results showed that 126 ferrets (62.7 %) in Japan were positive (Supplementary Table S1). Significant differences were statistically analyzed using Chi-squared and Fisher's exact probability tests. *P* values of <0.05 were considered to be statistically significant. The ratio of detection of FRCoV between diarrhea (75.9 %) and other symptoms (53.4 %) was significantly different. In particular, the difference was marked in ferrets aged more than 3 years, suggesting that FRCoV is the primary pathogen or exacerbating factor for diarrhea in ferrets. In addition, most ferrets aged less than 1 year (81.6 %) were infected with FRCoV, regardless of their clinical symptoms, suggesting that most ferrets are infected with FRCoV soon after birth. Importantly, some strains, Saitama-1 strain from ferret no. 22 and Aichi-1 strain from ferret no. 160, were detected by RT-PCR using CoV-consensus primers, but not FRCoV-specific primers, even though the latter can detect FRCoV with more sensitivity than CoV-consensus primers [14].

The nucleotide sequences of partial RdRp genes were determined using a BigDye Terminator Ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All sequences were deposited to the DNA Data Bank of Japan (DDBJ). A phylogenetic tree was constructed using the program MrBayes Ver. 3.2.2 [11] for MrModeltest analysis with a general time reversible (GTR) or WAG substitution matrices [15]. All trees were graphically represented using FigTree Ver. 1.4.2 [10]. The phylogenetic tree based on the partial RdRp genes showed that the FRCoV Saitama-1 and Aichi-1 strains belonged to a different cluster from other FRCoV strains (Fig. 1a).

In order to determine the 3′-terminal region of the Saitama-1 genome, further sequence analysis was performed. QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) and TaKaRa RNA LA PCR^{TM} Kit (AMV) Ver. 1.1 (TaKaRa, Shiga, Japan) were used to amplify each fragment of the Saitama-1 and Aichi-1 strains using the primer pairs listed in Supplementary Table S2. The 3′-terminal region, nt 6935-8271, of the Saitama-1 strain was amplified by 3′-RACE using TaKaRa RNA LA PCR^{TM} Kit (AMV) Ver. 1.1 (TaKaRa) according to the manufacturer's instructions. All sequences were deposited in the DDBJ. The nucleotide sequences from the S gene to poly A of the Saitama-1 strain (8271 bases) and N gene of Aichi-1 strain [1128 bases, 375 amino acids (a.a.)] were determined. The Saitama-1 strain had open reading frames (ORFs) encoding S (4308 bases, 1435 a.a.), ORF3 (744 bases, 247 a.a.), envelope (E) (249 bases, 82 a.a.), membrane (M) (798 bases, 265 a.a.), N (1128 bases, 375 a.a.), 3x-like (225 bases, 74 a.a.), and ORF7b (609 bases, 202 a.a.). The consensus transcription-regulating sequences (TRSs) of coronaviruses, 5′-CTAAAC-3′ [12], were observed upstream of each ORF, except ORF7b. Although we could not detect typical TRS of ORF7b, a possible TRS 5′-CTAAAG-3′ was observed upstream of ORF7b.

The nucleotide sequence from the S gene to poly A was compared among FRECV MSU-2, FRSCV MSU-1, and Saitama-1 strain using SimPlot Ver. 3.5.1 [5]. SimPlot analysis showed that the Saitama-1 strain was different in some regions, including the N, 3x-like, and ORF7b genes, when compared with other strains (data not shown). Surprisingly, the 3′-terminal in two-thirds of S genes in the MSU-2 strain was similar to that in the Saitama-1 strain, but one-third of S genes in the MSU-2 strain was similar to that in the MSU-1 strain (Fig. 2a). Alignment of S proteins among Saitama-1, MSU-1, and MSU-2 strains also indicated that the MSU-2 strain was similar to the MSU-1 strain in the *N*-terminal one-third of S protein and to the Saitama-1 strain in the *C*-terminal two-thirds of the protein

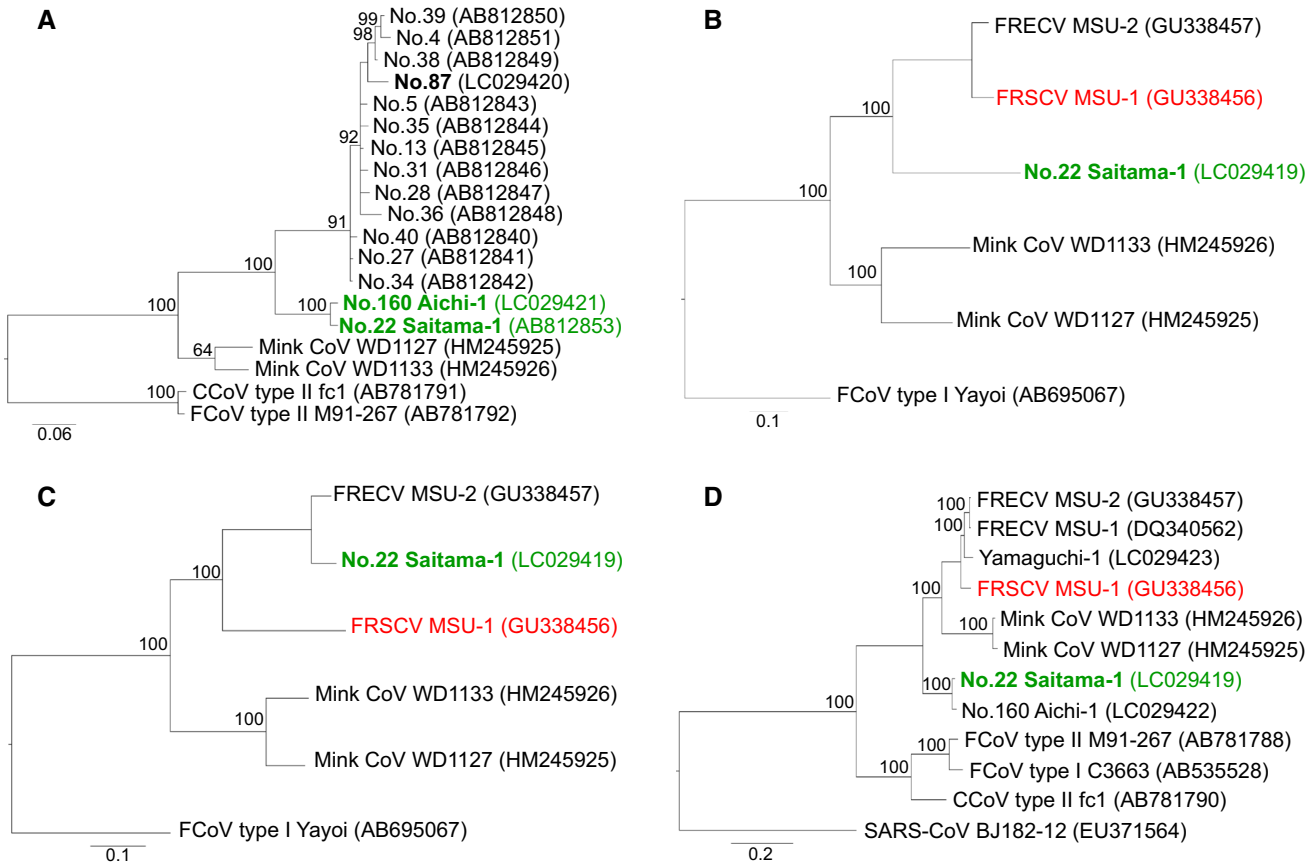


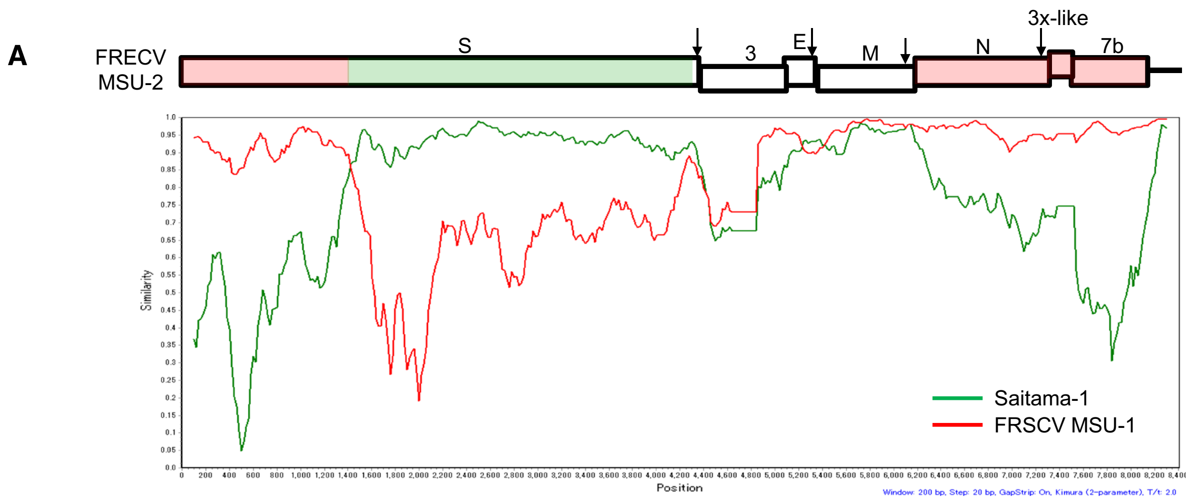
Fig. 1 Phylogenetic relationship among ferret coronaviruses. Phylogenetic trees were constructed based on nucleotide sequences of the partial RdRp gene (394 bp) (**a**), *N*-terminal one-third of S protein (a.a. 1-438 of Saitama-1 S protein) (**b**), *C*-terminal two-thirds of S protein (a.a. 439-1435 of Saitama-1 S protein) (**c**) and N protein (**d**). Posterior probabilities are indicated above branches in all trees. Sequences reported in this study are shown in *bold*

(Fig. 2b). In addition, a low similarity in nucleotide sequences between the MSU-1 and MSU-2 strains and the Saitama-1 strain was observed in the N, 3x-like, and ORF7b genes, but the E and M genes were highly conserved among all strains (data not shown).

Phylogenetic analysis was performed based on the *N*-terminal one-third of the S protein (a.a. 1-438 of Saitama-1 S protein) and the *C*-terminal two-thirds of the protein (a.a. 439-1435 of Saitama-1 S protein), indicating that the MSU-2 strain was more similar to the MSU-1 strain than the Saitama-1 strain in the *N*-terminal one-third of the S protein (Fig. 1b) and was more similar to the Saitama-1 strain than the MSU-1 strain in the *C*-terminal two-thirds (Fig. 1c). Furthermore, a phylogenetic tree based on the N protein indicated that Saitama-1 and Aichi-1 strains differed more from the other FRCoVs than from mink coronaviruses (Fig. 1d). These results indicate that the Saitama-1 strain possesses different S, N, 3x-like, and ORF7 genes than other FRCoVs, and that the E and M genes are conserved among FRCoVs. Therefore, we consider the Saitama-1 and Aichi-1 strains to comprise a novel FRCoV genotype. In addition, these results indicate that the MSU-2 strain emerged by recombination of the S protein between the MSU-1 and Saitama-1 strains. These recombination events often occurred among CoVs, resulting in the diversity of CoV genomes. Especially, the recombination of S protein caused the cross-species transmission or change of pathogenesis of SARS-CoV [4], porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCoV) type II [1], and feline coronavirus (FCoV) [13]. Novel CoVs must emerge and drastically evolve by these recombination events. Further investigation will thus be required to determine the evolution of CoV including FRCoV.

In conclusion, novel FRCoV strains (Saitama-1 and Aichi-1) were detected in Japan. These FRCoVs appear to have emerged by recombination events among other FRCoVs. This information will be useful for understanding the pathogenesis of FRCoV in ferrets.

A



B

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Saitama-1     .---FEMRT. .FIV.CYGLL QL---ACC.Y FN.TRFKNTS LIAVG.TK.F .H.VSN..S. ..NG.S.... ......E... D..S.GTDG. --VQ.DNLR. ..L.HEQG-- ----YAYNLS
FRECV MSU-2   MCNMIGITLL LLSCVSFVIT YENHTFSTDF SQCDSKAFIN TTLGYHYYLL DNFIKDYSKR LPTSSHVVLG DYFPTVQPWY GCVFNRSANG TMLSLHDIKA LYWDVKKT-- -NTTVSDCFV
FRSCV MSU-1   .......... .......... ....NS.... ......D... ..FVN..... .......... .......... .....L.... S......I.. .....N..R. ...YIRRIRY DTE...N...

Saitama-1     FAV..D...A I.ILNGDKFD GAAYGGLL.F .K-----D.S TIPHFSDN.T DS..IRS.RL .VDT.I.H.R .Y..K..S.. VV...E.... SYS.N.Y... NV..KTNHTI RPEHLSY...
FRECV MSU-2   VHLYGKPYTV TVKISAHGIT -SKDHNVMCI CEGKHEPYNY KCLTNTNSCG PGCIAHTFTA CANVSTCGDT LFGLRWSNSE LAAYLSGNVY RFRLANHWYN YATISDPDLY TVDNS--WWF
FRSCV MSU-1   .......... .......H.. -ASNR..... .S........ Q...EGRT.I D...E...P. .....I.... .......... .......... .......... .....Y.... RG.H.--L..

Saitama-1     ...RDLS..T .N.TGI.EII VN...EH.KG L.A......E ..F..PG.G. ........S. .......... .......... .T.....S.. VD.EN..... .....SG... .......V.D
FRECV MSU-2   NPVQSFTYYN VSKLSNGTVV YSNCTHNCAD YATNVFAVEK GGLIPSDFSF NNWFVLTNTS TVTSGKFVSS QPLLVNCLTP VPSFGEEALT LNFNQIPSQC NGATVNSSFD VVRFNLNFTS
FRSCV MSU-1   .......... .......... F....L.... RV.......E .....P.... .......... .........T .......... ....S..... .D........ .......... ..........

Saitama-1     S...S..KN. .T..V....E ..F..Y.... ..PRLY-E.T ....L.E.A. ....M....L S........N .R........ ......F.F. ....LS.I.. .......... ..........
FRECV MSU-2   GVVSASGTTF IALNTTGGVV LYLSCFNESQ TTSLFSGSGQ LPFGEHDGPL YCYVSYNETM FKFIGVLPPS VKEIAISKWG GVYVNGYNYF QTFPIDSVSF NLTTGSSGAF WTVAYTTFTD
FRSCV MSU-1   .......A.S L......... .H.......K ....L....F ......G... ....IH.D.. .......... .......... ...I...... .......L.. .......... .........E

Saitama-1     .......... .......... .......... .......... .......... .......... .......... VA..KLQ... ..S....... .......... .....R.E.H .......D..
FRECV MSU-2   VLLDVADTQI KSVVYCNSYV NDIKCSQLSA NLPDGFYPVS SLNLPNANLS FVTLPANFDH SYINVTGNVR MSVYGGTRIL SQGSNVTLYS GTVQSLCVNT SQFTLNFDSQ CSVEYGSTCT
FRSCV MSU-1   ...E.S.... ...T....HI .......M.E T........P .H....V.K. .....S..E. T.......LV LTYHSRPVVX --SG....SP TGT.TI.... .....F.... .TKFSQGR.D

Saitama-1     ...R...I.G .......... .......A.. ......LD.. .K...I...M ..D....... .G........ ....PNP... .....Y.... ........V. ..........
FRECV MSU-2   GAAMTEVRVD SGSCPFSFDK LNKHMTFEKL CISTVPVGDD CRFDLKARTV LGEFVFAHLY VSYKFGLDIT GLEVSDTGVK DLSNLHLNVC TEYNIYGHAG FGIIRQTNET LFGGLYYTSL
FRSCV MSU-1   .IGESKISIG A.N....P.. ..QYLS..T. .F..T.T.SA .A.EVTVQ.R FFTQ...N.F .T..Y...YL .VP.P.V... ...VVYQ... .S.S...... V....P..Q. .L........

Saitama-1     .......... ...T...... .......... .......... ......N... .......... .......M.- --........ .L........ .......... .......... ..........
FRECV MSU-2   SGDLLGFKNV TTGAVFSITP CEVSAQAAVI GGKIVGAITS VESDMLDLPH YIETPSFYYR SIYNYSATT- --YATSGMQK YFVNCTPVIT YSNMGVCENG ALVFVNITQS NSVAQPISTG
FRSCV MSU-1   .......... .....Y..V. .QL....... N.N....... ...SI...Q. ..V..Q...Y .......NFN VYSS.T.FD. .L........ ...L...... ....I.F... ENSV......

Saitama-1     .......... .......... .......... .......... .......... .......... .......... ......A... .......... D......... .......... ..........
FRECV MSU-2   NITIPSNFTI SVQVEYLQMS SELVSIDCAR YVCNGNARCN KLLTQYMSAC HTIEQALQTG VRLESLELES MISISDSALA IASVEQFNSS QHLNPVYSEG NNTIGGIYMD GLMNILPKKG
FRSCV MSU-1   .V........ .......... ..A....... ......P..D R....V.... .......S.S M...A..... ......N..Q L....A.... S..D.A.NAQ Y......... ..KDL..R.N

Saitama-1     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...I......
FRECV MSU-2   CANKHGSCRS TIEDLLFNKV VTSGLGTVDE DYKRCTKGLD IADLVCAQYH NGIMVLPGVV NSDKMAMYTA SLAGGITLGA LGGGLVAVPF ATAVQARLNY VALQTDVLQK NQKMLAASFN
FRSCV MSU-1   ..K...T.G. A..E...... ..T....... ......N... .........Y .......... .A........ .........V ......S... .......... .........Q ...I......

Saitama-1     .......... .......... .......... .......... .......... .......... .......... ...Q...... .......... .......... .......... ..........
FRECV MSU-2   QAIGNITQAF GKVNSAIQQT AKGLSTVAQA LTKVQDVVNS HGKALNQLTA QLQNNFQAVS SSIADIYYRL DELHADAQVD RLITGRLAAL NAFVTQTLTR MTQVRASRQL AKEKINECVR
FRSCV MSU-1   .......L.. .......E.. .Q.F...V.. .....N.... Q....Y.... ........I. ...E...H.. ..VN..V... .......... ......A..N RA........ ..D.......

Saitama-1     .......... .......... .......... .......... .........E .----..... .........N ..G....... ......F... .......... .......AIE ..........
FRECV MSU-2   SQSSRFGFCG NGTHLFSLAN AAPSGIMLFH TVLVPTSYTS VTAWSGICFD N----VGLIV KDVSLTLFKT HDDKFYLTPR TMYEPRVATS ADFVRINSCA TTFVNATVTD LPNIIPDYID
FRSCV MSU-1   .......... .......... ...R.V.... ...L....KT .M....V.AT STNKTM.F.. .........N ..GR...... .........M S...Q.E..T ........SE I.S.......

Saitama-1     ....I..... .......... .PI....... .......... ....A..... .I..G..D.. .......... ..TI...... .......... .......I.. .......... ..........
FRECV MSU-2   VNKTVQDMLE QYKPNWTVPN LSLDLFNLTY LNLTGEINDL ENRSVTLQQT VVELQALIAN INGTLVNLEW LNRVETYVKW PWWVWVIIGL ILLIALPMLL FCCLSTGCCG CCGCLTSCLA
FRSCV MSU-1   I.E.LK.... .........H FP..I..E.. ....S..SA. ..K.FS.... .K..EY..D. .......... ......F... .......... .......I.. .......... .....I....

Saitama-1     ........K. .......... ..
FRECV MSU-2   GCCKNSCKRP SYYEPIEKVH IN*
FRSCV MSU-1   ..F.....K. .....M.... ..
```

◀**Fig. 2** Recombination between Saitama-1 and the other FRCoV. **a** Schematic diagram of MSU-2 and SimPlot analysis of MSU-2 with Saitama-1 and MSU-1. *Green* and *red boxes* indicate regions similar to Saitama-1 and MSU-1, respectively. *Arrows* indicate TRS (5′-CTAAAC-3′). SimPlot analysis was carried out using the nucleotide sequence of the Saitama-1 strain from the S gene to poly A (8263 bases), the MSU-1 strain (8025 bases), and the MSU-2 strain (8309 bases). *Green* and *red lines* indicate Saitama-1 and MSU-1, respectively. **b** Alignments of S proteins of Saitama-1, MSU-1, and MSU-2. *Green* and *red boxes* indicate regions similar to Saitama-1 and MSU-1, respectively

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