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Artificial Recombination May Influence the Evolutionary Analysis of Newcastle Disease Virus▽

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The recombination rate in Newcastle disease virus (NDV) was as high as 10% in RDP analysis with full-length NDV genome sequences available in GenBank. We found that two NDV strains, China/Guangxi09/2003 and NDV/03/018, previously reported as recombinants, failed to show any evidence of recombination upon complete genome resequencing. Furthermore, we were able to reproduce artificial recombination by amplification of the M gene in a mixed sample of strains LaSota and ZJ1. It appears that the recombination of NDV is not as common as has been reported. NDV sequences in GenBank should be analyzed with caution during bioinformatic analyses for natural recombination events.

Newcastle disease (ND) is one of the most devastating diseases in poultry. The causative agent, Newcastle disease virus (NDV), is a member of the *Avulavirus* genus in the *Paramyxoviridae* family (2, 9, 11, 24). The NDV genome consists of approximately 15-kb-long nonsegmented single-stranded negative-sense RNA that codes for six proteins, including nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin neuraminidase (HN), and polymerase protein (L) (2, 6, 28). Although NDV has only one serotype, substantial antigenic and genetic diversity have been previously recognized (2, 19). According to earlier reports, at least 10 genotypes (genotypes I to X) have been described for NDVs (4, 29).

The main dynamics of evolution in nonsegmented RNA viruses either are due to the inherent error rate of the RNA-dependent RNA polymerase or occur as a result of recombination (30). While polymerase error is believed to be the main driving force for NDV evolution (30), it has been established that recombination in nonsegmented negative-sense RNA viruses, including NDV, is rare (1, 7). Indeed, during 2005 to 2009, we characterized more than 100 NDV strains in our laboratory (20, 43), but no recombinant strains were detected on the basis of the analysis of F and HN gene sequences. However, in recent years, more and more recombination events have been reported for NDVs, with the recombination occurring throughout the whole genome (7, 8, 15, 30, 36, 47, 44, 46). Han et al. and Zhang et al. even reported recombinants of NDVs that involved multiple genotypes (15, 46).

This controversy has prompted us to investigate whether the recombination events in NDVs are as common as has been reported. Eighty complete genomic sequences of NDV retrieved from GenBank (Table 1) were edited using BioEdit version 7.0.0 and aligned using ClustalX version 1.83 software (41). To detect recombination events over the whole genome of NDVs, we used different statistical methods included in the RDP3.42 software package (17): RDP (21), Geneconv (33), Bootscan (22), Maxchi (40), Chimaera (35), SiScan (12) and 3Seq (5). As different algorithms might not be completely consistent with each other, any breakpoint supported by five or more methods with P values $\leq 10^{-5}$ was set as a positive recombination signature. KBNP, a chimeric vaccine strain (GenBank accession number EU140955), was used as a control to evaluate the prediction capability of the program. Our analysis indicated a recombination rate as high as 10%. There are at least 8 recombinants in the 80 NDVs, involving 15 recombination events (Fig. 1). These events were detected throughout the whole genome but more frequently in M, F, and HN genes. Moreover, 5 of the 8 recombinants were involved in multirecombination events. Interestingly, these recombinants were mainly isolated in China and the recombination events were concentrated in viruses of genotypes I, II, III (three genotypes of viruses widely used as vaccines), and VII (a predominant genotype currently circulating worldwide). Among these recombinants, virus strain China/Guangxi09/2003 (DQ485230) was a putative daughter virus of genotype VII virus FWM (GU564399) and genotype III virus Mukteswar (EF201805), as detected by RDP ($P = 2.528 \times 10^{-70}$), Geneconv ($1.478 \times 10^{-68}$), BootScan ($1.730 \times 10^{-70}$), MaxChi ($2.539 \times 10^{-13}$), Chimaera ($1.628 \times 10^{-13}$), and SiScan ($2.267 \times 10^{-17}$). Another virus, NDV/03/018 (GQ338309), appeared to have arisen from a recombination event between genotype VII virus NDV/03/044 (GQ338310) and genotype III virus JS-7-05 (FJ430159), as identified accordingly to analysis by RDP ($P = 1.862 \times 10^{-35}$), Geneconv ($3.859 \times 10^{-21}$), Bootscan ($7.608 \times 10^{-35}$), MaxChi ($1.044 \times 10^{-08}$), Chimaera ($3.401 \times 10^{-07}$), and SiScan ($3.344 \times 10^{-08}$). The maximum-likelihood (ML) trees were constructed using PhyML and the GTR+I+G model and selected by jModelTest (13, 14, 34), and the approximate-likelihood-ratio test values were examined for branch support (3, 14). The tree based on the full-length genomes indicated that these two potential recombinants were affiliated with genotype VII (Fig. 2A), while the putative recombination regions clustered into genotype III (Fig. 2B).

Using strains China/Guangxi09/2003 and NDV/03/018 as examples, we attempted to validate whether the unexpected high

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TABLE 1. Sequences of NDVs used in this study

Accession no.	Strain	Country	Genotype	Reference[a]
AY562991	Ulster-67	Ireland	I	
AY935489	01-1108	AUS	I	18a
AY935490	02-1334	AUS	I	18a
AY935491	98-1154	AUS	I	18a
AY935492	98-1249	AUS	I	18a
AY935493	98-1252	AUS	I	18a
AY935494	99-0655	AUS	I	18a
AY935496	99-0868lo	AUS	I	18a
AY935497	99-1997PR-32	AUS	I	18a
AY935498	99-1435	AUS	I	18a
AY935500	I-2 progenitor	AUS	I	18a
DQ097394	PHY-LMV42	Germany	I	8a
GQ918280	BHG	Sweden	I	30b
HM063422	D3	China	I	
HM063424	R8	China	I	
HM125898	WDK/JX/7793/2004	China	I	
AF077761	La Sota		II	9
AF309418	B1	USA	II	
AF375823	B1 isolate Takaaki	USA	II	30c
AY225110	HB94 isolate V4	China	II	
DQ060053	A11-ND026	China	II	
EU140955	KBNP		II	7b
EU289028	VG-GA	USA	II	33b
EU546165	JL-1	China	II	
FJ386392	NDV01	China	II	
FJ386393	NDV02	China	II	
FJ386394	NDV03	China	II	
FJ386395	NDV04	China	II	
FJ386396	NDV05	China	II	
FJ939313	NDV-Chicken	Egypt	II	30a
GQ994433	XD/Shandong/08	China	II	46
GU978777	APMV-1-U.S.-GB	USA	II	33a
Y18898	Clone30		II	37b
EF201805	Mukteswar	China	III	
FJ430159	JS-7-05	China	III	37a
FJ430160	JS-9-05	China	III	37a
AY741404	Herts/33	Holland	IV	9a
EU293914	Italien	China	IV	42a
AY562986	Anhinga	USA	V	
AY562987	U.S.(CA)/211472/02	USA	V	
AY562990	Largo	USA	V	
AJ880277	Pigeon paramyxvirus-1	Hunga	VI	41a
AY562988	U.S.(CA)/1083	USA	VI	
AY562989	Italy/2736/00	Italy	VI	
FJ410145	PPMV-1/New York/84	USA	VI	
FJ410147	PPMV-1/Maryland/84	USA	VI	
FJ766526	JS/07/22	China	VI	
FJ766527	JS/07/16	China	VI	
FJ766528	NDV05-029	China	VI	
FJ766529	ZhJ-3/97	China	VI	
FJ766530	JS-07-04	China	VI	
FJ766531	JS-07-03	China	VI	
GQ338311	ND/05/028	China	VI	
GQ429293	Italy/2736/00	Italy	VI	10
HM063425	P4	China	VI	
HM063423	W4	China	VI	
AF431744	ZJ1	China	VII	17a
AF473851	SF02	China	VII	48
AY562985	Indonesia/14698/90	Indonesia	VII	
DQ485229	China/Guangxi7/2002	China	VII	
DQ485230	China/Guangxi09/2003	China	VII	
DQ485231	China/Guangxi11/2003	China	VII	
DQ486859	GM strain	China	VII	
DQ659677	NA-1	China	VII	
DQ839397	KBNP/Korea	Korea	VII	7a
EU167540	SRZ03	China	VII	36
FJ872531	China(Fujian)/FP1/02	China	VII	
GQ338309	NDV/03/018	China	VII	

Continued on following page

TABLE 1—*Continued*

Accession no.	Strain	Country	Genotype	Reference[a]
GQ338310	NDV/03/044	China	VII	
GQ849007	JSD0812	China	VII	
GU143550	Go/CH/HLJ/LL01/08	China	VII	
GU564399	FMW	China	VII	
GQ994434	QG/Hebei/07	China	VII	46
FJ751918	QH1	China	VII	
FJ751919	QH4	China	VII	
FJ436302	F48E8	China	IX	37
FJ436303	ZJ/1/86	China	IX	37
FJ436304	FJ/1/85	China	IX	37
FJ436305	JS/1/97	China	IX	37
FJ436306	JS/1/02	China	IX	37

[a] A reference number is given if available.

recombination rate was due to inaccuracy of some of the NDV sequences deposited in GenBank. The two viruses were resequenced and analyzed in this study. Viruses were subjected to plaque purification using primary chicken embryo fibroblasts (16). Viral RNAs were extracted from infective allantoic fluid by the use of TRIzol reagent (Invitrogen, Carlsbad, CA). The putative recombination regions of both viruses were amplified using specific primers (available upon request). PCR products were sequenced using an ABI Prism BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). ML trees based on the putative recombination regions of our newly obtained sequences were constructed. Their positions in the new trees were consistent with those in the trees constructed from full-length sequences (Fig. 2A and C). However, the putative recombinants detected with the old versions of sequences in GenBank (DQ485230 and GQ338310) were not observed, indicating that these recombinants are artificial. As both viruses were detected as recombinants of genotype VII and genotype III viruses, we then tried to identify the presence of genotype III viruses in original samples. Using genotype III-specific primers (available upon request), we were able to amplify genotype III sequences from both samples. These genotype III sequences are identical to the putative recombination regions in the old versions of sequences in GenBank. These data demon-

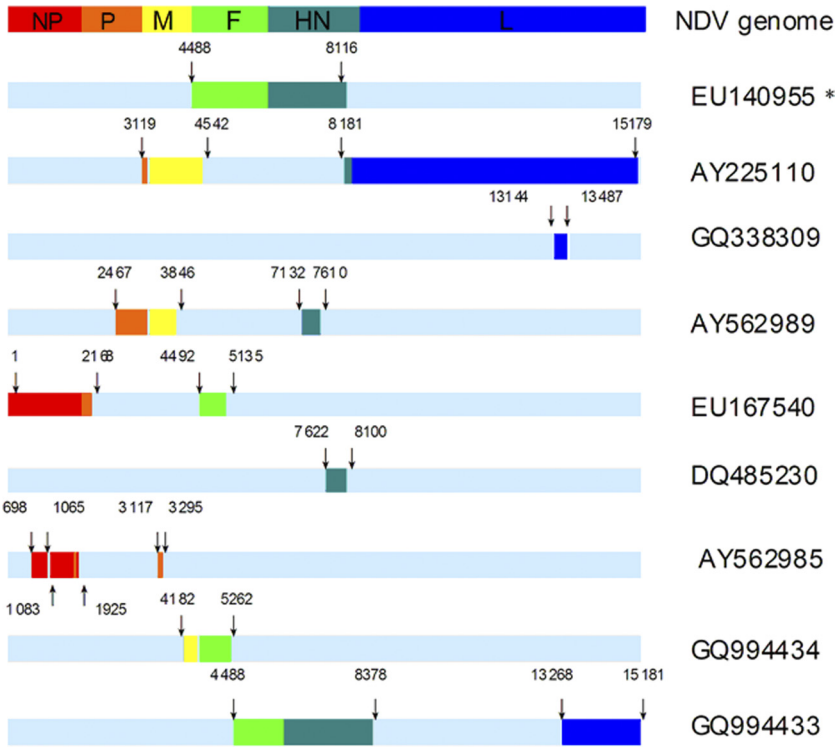

FIG. 1. Recombination events detected simultaneously by five or more methods using the RDP3.42 program with P values $\leq 10^{-5}$. The nucleotide positions of breakpoints in the whole genome are shown above or below the bars. Red, orange, yellow, green, cyan, and blue bars indicate NP, P, M, F, HN, and L genes, respectively. The asterisk indicates KBNP (EU140955), a chimerical vaccine strain that has the genotype II Lasota vaccine strain backbone and the F and HN genes from a genotype VII virus, which was used as a control to evaluate the prediction capability of the program.

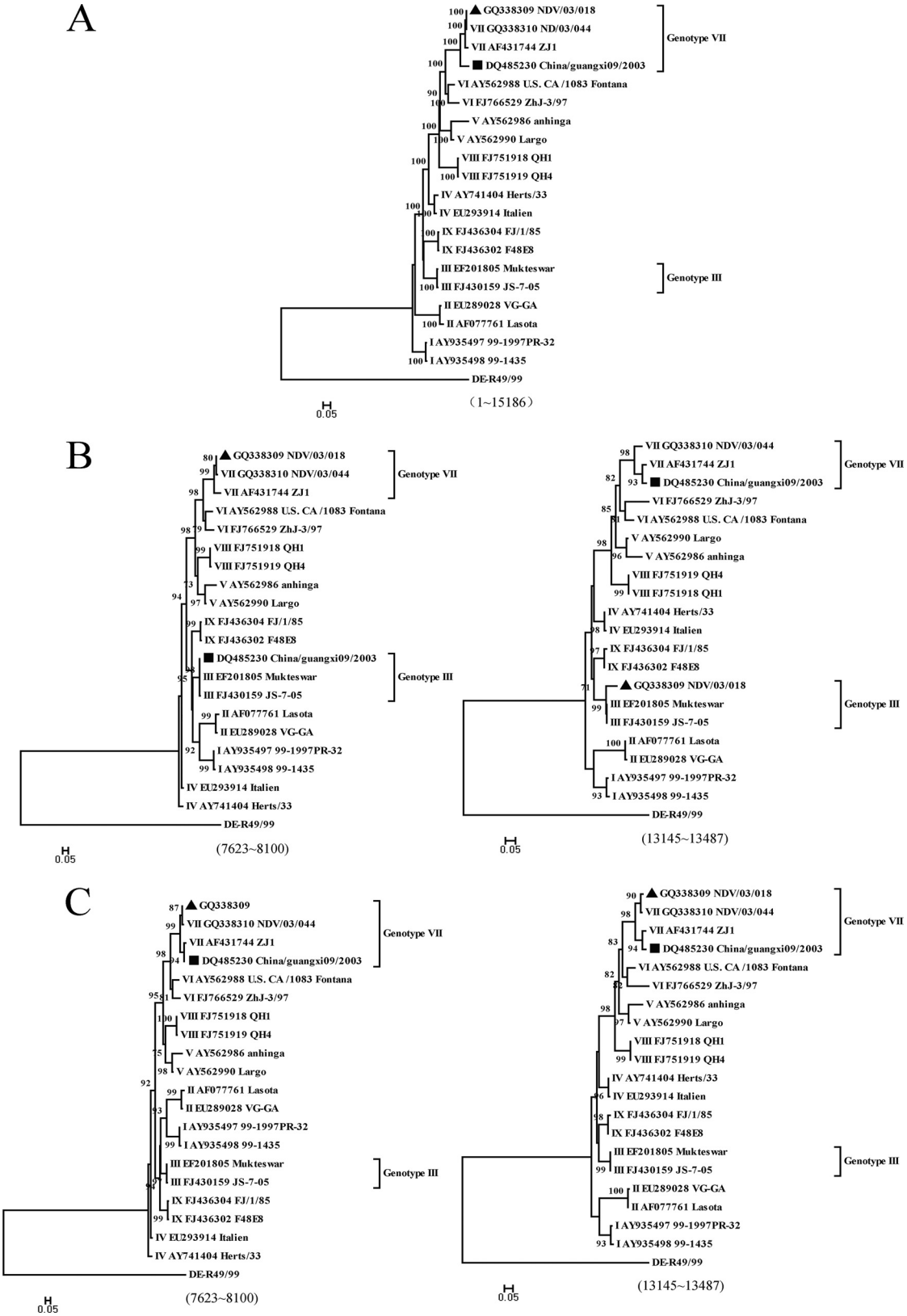

FIG. 2. Maximum-likelihood trees based on whole-genome and putative regions of NDVs. The scale bars represent the numbers of substitutions per site, and the numbers at each node represent values from approximate-likelihood-ratio tests for branch (Shimodaira-Hasegawa-like) support. Support values under 70 were removed. Putative recombinants are marked by either a black square for China/Guangxi09/2003 or a black triangle for NDV/03/018. (A) Phylogenetic trees of NDV strains based on complete genomic sequences showed that the two putative recombinants are of genotype VII. (B) Phylogenetic trees based on the putative recombination regions showed that they belong to genotype III. (C) Phylogenetic trees based on the revised sequences showed that the putative recombinant regions are affiliated with genotype VII, indicating that the two old versions of sequences in GenBank represent artificial recombinants.

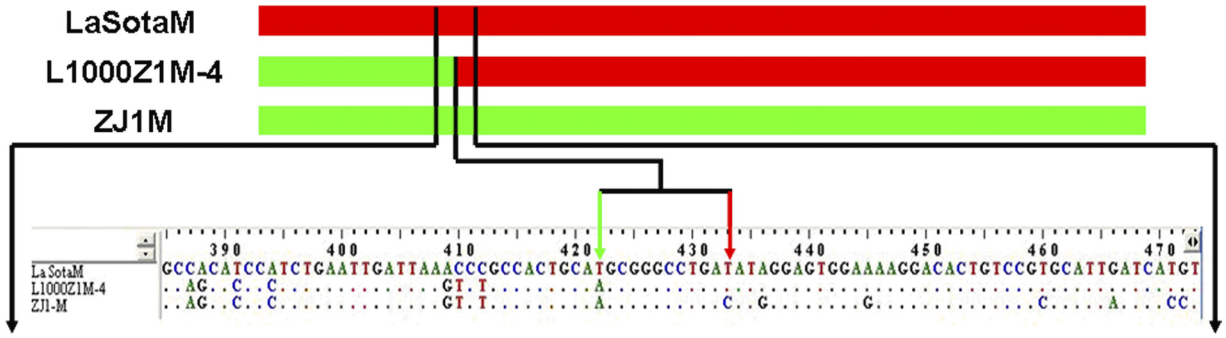

FIG. 3. Artificial recombinant fragments were amplified from a mixed-NDV sample. LaSotaM and ZJ1M represent M gene sequences of Lasota and ZJ1. LaSota sequences and ZJ1 sequences are indicated by green and red bars, respectively. LaSota and ZJ1 genome sequences were identified in one clone (L1000Z1M-4). The fragment between the green and red arrows denotes a potential template switching position.

strated that the original China/Guangxi09/2003 and NDV/03/018 isolates were mixtures of genotype VII and genotype III viruses and that the genomic sequences deposited in GenBank previously might be a reflection of artificial recombination. It is very likely that these two recombinants were generated through RNA template switching between genotype VII and III components.

To provide evidence for a potential artificial recombination resulting in a mosaic sequence, we performed reverse transcription-PCR (RT-PCR) amplification of the M gene from a mixed sample of LaSota (allantoic fluid; HA titer, 512) and ZJ1 (allantoic fluid; HA titer, 128) viruses, which represent genotypes II and VII, respectively. The volume ratio of the former to the latter was 1,000:1. Parental LaSota and ZJ1 viruses were used as controls. A pair of primers, 5′-AGGGCAGAGCCAARACARTAC-3′ and 5′-CGCRGTTTGRCTCCAGAGTAT-3′, were used for the amplification. The amplification was performed in a 25-μl total reaction volume with 1.5 U *Taq* DNA polymerase (Fermentas, CA). PCR products were cloned into pGEM-T vector (Promega), and multiple clones were sequenced. Clones carrying either genotype II or VII sequences were identified. Notably, the two genotype sequences could also be identified in the same clone, e.g., the clone designated L1000Z1M-4 (Fig. 3), which was identified as a recombinant by all of the seven statistical methods with P values $< 10^{-5}$ (data not shown). These results suggest that artificial recombination events can be easily induced with samples containing mixed virus genotypes or strains, probably through polymerase template switching during the PCR procedure.

Our study clearly demonstrated that some NDV sequences in GenBank are inaccurate and may affect the bioinformatic analysis for NDV evolution. Consistent with these findings, we noticed that another NDV strain, Italy/2736/00, possesses two accession numbers (AY562989 and GQ429293) in GenBank. The virus was characterized as a recombinant based on the originally uploaded sequence (10). Later in 2009, the author submitted a new version of the sequence (GQ429293) and indicated the absence of recombination in the virus and that the earlier sequence represented an artificial recombination. In 2003, F and HN gene sequences of JS2/98/Go were deposited in GenBank with assigned accession numbers AF456439 and AF456430, respectively. The phylogenetic analysis of these sequences indicated that this virus was a recombinant of F and HN genes from genotypes VI and VII. However, subsequent resequencing confirmed this virus to be an entirely genotype VII strain (data not shown). We believe that the actual recombination rate in NDVs is lower than that deduced by using NDV sequences from GenBank.

Currently, lentogenic NDV strains Hitchner B1, Australia V4, and LaSota and mesogenic strain Mukteswar are used as live vaccines worldwide (19, 25, 26, 42), especially in Asia. However, infections caused by virulent NDV strains frequently occur in poultry despite vaccination, providing a good opportunity for the coexistence of virulent and vaccine NDV strains. Due to the propensity of *Taq* DNA polymerase to slip during the elongation step in PCR (18, 23, 27, 31, 32, 38, 39, 45), molecular work using RNA templates extracted from a field virus sample (which may contain nucleic acids from various NDV strains) is likely to produce artificial recombination fragments by template switching during the process of PCR. On the other hand, the cross-contamination of PCR products, which was pointed out earlier as a frequent occurrence in NDV research laboratories (1), might also cause artificial recombination. We suggest careful verification of NDV sequences while performing bioinformatic analysis for evaluation of virus evolution. In addition, field samples should be subjected to plaque purification before sequencing to ensure the accuracy of NDV sequence data deposited in GenBank.

Nucleotide sequence accession numbers. We have recently updated sequences of China/Guangxi09/2003 and NDV/03/018 in GenBank under accession numbers JF343539 and JF343538, respectively.

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