Genetic analyses of the fusion protein genes in human parainfluenza virus types 1 and 3 among patients with acute respiratory infections in Eastern Japan from 2011 to 2015

（2011 年から 2015 年の東日本における急性呼吸器感染症患者のヒトパラインフルエンザウイルス 1 型および 3 型の融合タンパク質遺伝子の遺伝学解析）

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Abbreviations

ARI: acute respiratory infections（急性呼吸器感染症）

FEL: fixed effects likelihood（エフイーエル）

HN: hemagglutinin–neuraminidase（ヘムアグルチニン-ノイラミニダーゼ）

HPIV: human parainfluenza virus（ヒトパラインフルエンザウイルス）

HRSV: human respiratory syncytial virus（ヒト RS ウイルス）

IFEL: internal fixed effects likelihood（アイエフイーエル）

MEGA: molecular evolutionary genetics analysis（分子進化遺伝学的分析）

ML: maximum likelihood（最尤法）

RT-PCR: reverse transcription polymerase chain reaction（逆転写ポリメラーゼ連鎖反応）

SLAC: single likelihood ancestor counting（スラック）

Introduction

Human parainfluenza virus (HPIV) type 1 (HPIV1) and type 3 (HPIV3) belong to the genus *Respirovirus* and the family *Paramyxoviridae*. These viruses are responsible for a myriad of acute respiratory infections (ARIs), including the common cold, croup, bronchitis, bronchiolitis, and pneumonia [18]. Previous epidemiological studies suggest that primary HPIV1 and HPIV3 infections have usually occurred in over 80% of children by the age of 5 years [32, 33]. Some infants with primary HPIV infection may develop severe clinical symptoms, including pneumonia accompanied with wheezing [10, 23]. Furthermore, HPIV infection, seasonal influenza, and human respiratory syncytial virus (HRSV) infections may occur throughout life [6, 10, 31]. Although the epidemiology of HPIV is unclear, previous epidemiological research suggests that HPIV1 and HPIV3 are the dominant causes of primary infection in infants, rather than HPIV2 and HPIV4 [18, 24, 31].

The HPIV1 and HPIV3 genomes translate into 7 or 8 proteins, respectively [18, 24]. Of these, two structural proteins—the fusion (F) protein and the hemagglutinin–neuraminidase (HN protein)—have been identified as the major antigens [14, 18]. Indeed, it has been suggested that the HPIV F protein is pivotal to infection in the host cell [17, 41, 43, 44]. The F protein may bind to host cells to facilitate infection, which also acts on syncytium formation between infected cells and F proteins of other viruses, such as HRSV, measles virus, mumps virus, and human metapneumovirus [4, 6, 11].

Some molecular epidemiological studies suggest that HPIV1 can be further divided into three major genetic clusters or clades [2, 22, 29]. HPIV3 can also be classified into three distinct clusters (A, B, and C) with the subdivision of cluster C further divided into five distinct subclusters (C1-5) [1,8, 37]. In addition, it is not clear whether the evolution of HPIV3 is related temporally

or geographically, or if amino acid diversity reflects both the functional and structural constraints on the protein, as well as the epidemiologic features of an HPIV3 infection [48]. Although it is important to understand the properties of the *F* gene and its protein product in HPIV1 and HPIV3 strains, these details are still unknown. Therefore, we performed genetic analyses of the *F* gene in HPIV1 and HPIV3 in patients with ARIs in Eastern Japan (Aomori, Iwate, and Gunma prefectures) between 2011 and 2015.

Method

1. Samples and patients

The study protocol was approved by the National Institute of Infectious Disease Ethics Committee (No. 495). Samples were obtained by the local health authorities of Aomori, Iwate, and Gunma prefectures for the surveillance of viral diseases in Japan between April 2011 and March 2015. A total of 2,069 pharyngeal or nasopharyngeal swab samples were collected from patients with ARIs, including upper respiratory illness, asthmatic bronchitis, bronchitis, and pneumonia. Informed consent was obtained from subjects (or from the parents of underage subjects) for sample donation. Either HPIV1 or HPIV3 was detected in 72 of the included patients (72/2,069; 3.5%).

2. RNA extraction, reverse transcription polymerase chain reaction, and sequencing

Viral RNA was extracted from clinical samples using a QIAamp Viral RNA Mini kit (Qiagen). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a QIAGEN® OneStep RT-PCR Kit (Qiagen), using PCR primers for cDNA synthesis and amplification. The detection of HPIV1 and HPIV3 were carried out as previously described 3). To analyze the full length sequences of the HPIV1 and HPIV3 *F* genes with the primed walking method, some primer sets were newly designed by Primer Express version 1.5 software (Applied Biosystems) [16]. The full length nucleotide sequence of the *F* gene spanned bases 5,088–6,754 (1668 nt) of the prototype Washington/1964 strain in HPIV1 and the 14702 strain, 5,072–6,691 (1620 nt) in HPIV3. The primer sequence data are shown in Table S1 [2, 39, 50]. The PCR products were purified with a QIAquick PCR

Table S1 Primer sets HPIV1 and HPIV3

Subtype	Positions*	Primer (5'-3') **	Sequence (5'to 3')
HPIV1	4695–4718	4695+	GGGAAAATCAGGAAGATATAAGTC
	5442–5466	5442-	CTCCTAGTGCTATGGTACCAATGAC
	5346–5370	5346+	CTGAAGGATGCCTTGGATCTTCAGG
	6111–6134	6111-	GGATCTCTAGGACATATATATGC
	6012–6034	6012+	GAAGGAGAAGAATGGCATGTCGC
	6483–6504	6483-	CTAGGAAATTTGTGGCAGATGC
	6277–6299	P1F6277-24+ ***	GCGGGACAAACAGAATACCAGTGA
	6937–6960	6937-	CATTTCGGGTTGTAGACCAATATG
HPIV3	4724–4750	FS-4724-4750+ ****	TGCAATTTTCCAACCTTCTTTACCTGG
	5469–5492	5469-	CTGCTTGGCTTCAACCAGAGCAAC
	5405–5425	5405+	CTTTGGAGGGGTAATTGGAAC
	6145–6166	6145-	GGACATTRGGATATGTTTCCTG
	6033–6055	6033+	GGGGCATTTCTAGGTGGAGCAG
	6735–6758	6735-	GAGTTGCGTAACTTTACTCCTAAG
	6647–6673	HNS-6647-6673+ ****	AAATCGAGTGGATCAAAATGATAAGCC
	7085–7110	FA-7085-7110- ****	AAGAAGCCTTGTATTCACTCCTGACT

*Nucleotide position was based on the sequence of HPIV1 (Washington 1964, GenBank accession no. AF457102) or HPIV3 (14702-2008, GenBank accession no. EU424062*****). Abbreviations: HPIV1, Human parainfluenza virus type 1; HPIV3, Human parainfluenza virus type 3.

**+ for Forward primer; - for reverse primer

*** Beck et al., 2012

**** Yang et al., 2011

*****Roth et al., 2009

Purification Kit (Qiagen) and then sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), using newly designed primer sets. Sequence analysis was performed on an ABI 3500 Genetic Analyzer (Applied Biosystems). The GenBank accession numbers of the nucleotide sequences obtained in the present study are LC076586–LC076603, LC076605, LC076622, LC076624–LC076630, and LC078993.

3. Phylogenetic analysis by the maximum likelihood method

Phylogenetic analysis of the nucleotide sequence of the HPIV1 and HPIV3 *F* genes were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 6 [45]. Evolutionary distances were estimated according to Kimura's two-parameter method, and the phylogenetic tree was constructed with the maximum likelihood (ML) method [20, 42]. We used Kakusan4 [46] to select an appropriate substitution model, and GTR + Γ was selected. The reliability of the tree was estimated with 1000 bootstrap replications using the GTR +Γ substitution model [13]. To construct the phylogenetic tree, we collected comprehensive data on the HPIV1 and HPIV3 *F* gene sequences from GenBank. The GenBank accession numbers of the strains were AB012132, AB736166, AF457102, EU326526, EU424062, FJ455842, JQ901971–JQ901980, JQ901983–Q901997, Q901999–JQ902003, JQ902005, JQ902006, JQ902008, JQ902010, KF530196–KF530198, KF530203, KF530205, KF530211, KF530212, KF530217, KF530221, KF530225, KF530226, KF530229, KF530230, KF530232–KF530234,KF530236, KF530241–KF530243, KF530245, KF530249–KF530253, KF530256, KF530257, KF687307, KF687310–KF687315, F687318–KF687321, KF687323, KF687325–KF687328,KF687330, KF687332–KF687334, KF687336,

KF687340, KF687343, KF687344, KF687346–KF687349, KF687351–KF687354, KF687357, KF687358, KJ672527, KJ672530–KJ672533, KJ672535–KJ672539, KJ672541, KJ672542, KJ672545, KJ672547, KJ672549, KJ672553–KJ672555, KJ672559, KJ672560, KJ672568, KJ672574–KJ672576, KJ672579, KJ672582, KJ672584, KJ672586, KJ672588, KJ672591, KJ672593, KJ672595, KJ672596, KJ672601, KJ672604, KJ672605, KJ672607–KJ672610, KJ672612–KJ672616, KJ672618, and S82195.

4. Pairwise distance calculation

To assess the genetic distances of all present strains, we calculated pairwise distances (*p*-distance) for the HPIV1 or HPIV3 strains (both the current and reference strains), as previously described [29, 30].

5. Selective pressure analyses

To evaluate the action of selective pressure on the *F* gene across all HPIV1 or HPIV3 strains, we estimated the rates of non-synonymous (*dN*) and synonymous (*dS*) changes at amino acid sites by conservative single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and internal fixed effects likelihood (IFEL) methods using DataMonkey (http://www.datamonkey.org) [35]. Positive/negative selection ($dN>dS$) was determined by *p*-values ≤0.05 for SLAC, FEL, and IFEL.

6. B cell linear epitope analyses

To examine the relationship between the positive selection sites and epitopes, the linear B cell epitopes of the HPIV1 and HPIV3 strains were predicted using Epitopia, BCPred, FBCPred, BepiPred, and

Antigenic as previously reported [7, 21, 25, 38, 40]. We used these tools under default conditions, with the exception of the epitope length. In the present study, the epitope lengths predicted by Antigenic and FBCPred were set at 10-mer amino acids, while the epitope length predicted by BCPred was set at 12-mer amino acids. We accepted the sites as the linear B cell epitopes which were inferred by three or more methods and with more than 10-mer consecutive amino acids as previously reported [21].

7. Mapping of amino acid substitutions of the F protein in HPIV1 and HPIV3

To assess the relationships between amino acid substitutions and antibody reactivity against the F protein, we mapped the amino acid substitutions of the F protein of the present strains, as previously described [19]. The models were constructed using MODELLER v9.16 using the Washington 1964 strain and the Wash/47885/57 strain as templates for HPIV1 and HPIV3, respectively [49]. Homology modeling for HPIV1 and HPIV3 was based on the crystal structure of 1ZTM (Protein Data Bank accession number for the HPIV3 F protein), and the energy of each constructed model was minimized using Swiss PDB Viewer v4.1 [12]. Then, the models were evaluated by Ramachandran plots produced using the RAMPAGE server [26]. Finally, the models were colored using Chimera v1.10.2, and the amino acid substitutions corresponding to the prototype strains were mapped on the predicted models [34]. Furthermore, we also mapped the neutralization-related amino acids for the F protein in HPIV3 as suggested by a previous report [5].

Results

1. Detection of HPIV1 and HPIV3 in this study

We collected 2,069 pharyngeal or nasopharyngeal swab samples from patients with ARIs and detected 19 strains of HPIV1 (0.92%) and 53 strains of HPIV3 (3.5%). These HPIV1- or HPIV3-positive patients were variously diagnosed with upper respiratory illness, lower respiratory illness, bronchitis, bronchiolitis, or pneumonia. HPIV1 strains were typically detected in patients aged 21.8 ± 36.0 years (range: 1 month to 88 years), and HPIV3 were typically detected in patients aged 2.2 ± 2.4 years (range: 1 month to 13 years). No differences were seen in the male-to-female ratios.

2. Phylogenetic analysis, nucleotide identities, and p-distance in HPIV1 or HPIV3 strains

To assess the identified HPIV1 and HPIV3 strains phylogenetically, we constructed the phylogenetic trees according to the *F* gene nucleotide sequences of HPIV1 and HPIV3 using the ML method (Fig. 1a and 1b). The identified HPIV1 strains could be classified into two clusters (cluster II and III), and HPIV3 were classified into cluster C. In addition, HPIV3 was further classified into distinct 4 subclusters (C1, C2, C3, and C5). The nucleotide sequence identities were 93.8–100% and 93.2–100%, respectively. In addition, we calculated the *p*-distance among the HPIV1 and HPIV3 strains to be 0.018 ± 0.011 and 0.026 ± 0.018, respectively (Fig. 2a and 2b).

3. Positive/negative selection sites in the F gene of HPIV1 or HPIV3

We analyzed the positive and negative selection sites in the strains using SLAC, FEL, IFEL methods, and calculated the *dN*/*dS* values. The details of

these analyses are presented in Tables 1 and 2. For HPIV1, we found one positive selection site at amino acid 5 by the FEL method and one positive selection site at amino acid 8 by the IFEL method. For HPIV3, we found one positive selection site at amino acid 108 by the IFEL method. Many negative selection sites were detected by all methods for both strains. More than 10 and 60 negative selection sites were estimated in HPIV1 and HPIV3, respectively.

4. Linear B cell epitopes analyses

The predicted linear epitopes are shown in Table 3, Fig. 3, and Fig. 4. As shown in Table 3, in the HPIV1 and HPIV3 prototype strains, four and five epitopes were predicted, respectively. Among them, a common sequence of seven amino acids (YICPxDP) was found in both of the strains. The common predicted epitope motif was located on the head of the F protein (Figs. 3 and 4), which corresponded to the positions at amino acids (aa) 341-347 in the F protein of the HPIV1 prototype strain and at aa 338-344 of the HPIV3 prototype strain, respectively. Moreover, a predicted epitope of HPIV3 was involved with the positive selection sites (aa 108), while the HPIV3 epitopes did not correspond to the neutralization reactive sites of HPIV3 (aa 73, aa 396-398). The epitopes in the HPIV1 F protein were not involved with the positive selection sites.

5. Amino acid substitutions of the F protein in the predicted models

The predicted structural models of the HPIV1 and HPIV3 F proteins are shown in Fig. 3 and Fig. 4, respectively. The HPIV1 model shows aa22-94 and aa142-484 corresponding to the F protein of the HPIV1 prototype strains (Washington 1964 strain), and the model for HPIV3 shows aa25-97 and aa145-487 corresponding to the protein of the HPIV3 prototype strains

(Wash/47885/57 strains). Ramachandran plots showed that substitutions in each cluster on the surface of the F protein are indicated in red. The other HPIV1 strains in clusters II and III had 11 amino acid substitutions and 29 amino acid substitutions corresponding to the HPIV1 prototype strain, respectively (Table S2). Among them, five or 11 amino acid substitutions were estimated on the surface of the F protein for the HPIV1 clusters II and III, respectively (Fig. 3). Moreover, our HPIV3 strains had 34 amino acid substitutions corresponding to the HPIV3 prototype strain (Table S3). In addition, there were nine amino acid substitutions on the surface of the protein. Of these, R73K could affect resistance to the neutralization by monoclonal antibodies and was estimated to be within the HPIV3 cluster C strains (LC76627) [5].

Fig. 1a
Phylogenetic trees of the *F* gene for HPIV1 (a) constructed by the maximum likelihood (ML) method.

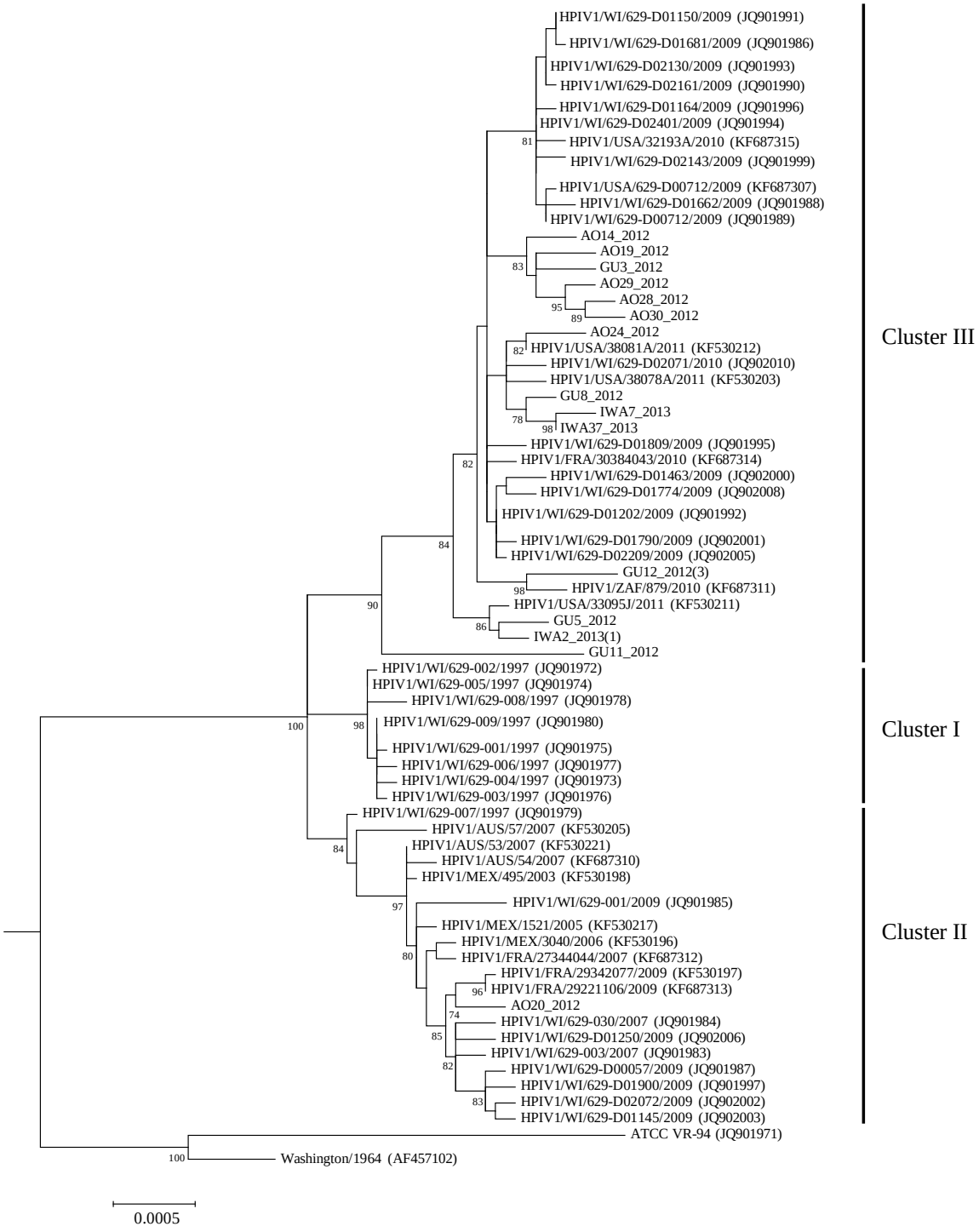


Fig. 1b
Phylogenetic trees of the *F* gene for HPIV3 (b) constructed by the maximum likelihood (ML) method.

Fig. 2. Distribution of pairwise distances based on the nucleotide sequences of the *F* gene in HPIV1 (a) and HPIV3 (b).

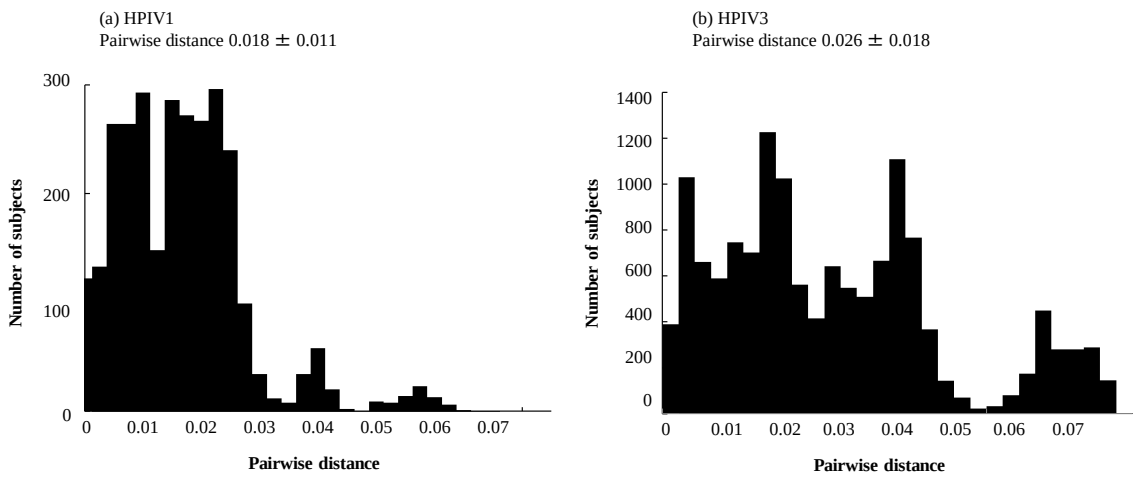


Table 1. Positive selection sites in the HPIV1 and HPIV3 *F* genes

Virus	aa position	Change	SLAC	FEL	IFEL
HPIV 1	5Glu	Lys, Gly, Asp		x	
	8Leu	Ile, Phe			x
HPIV 3	108Glu	Lys, Gly			x
	108Lys	Glu, Gly			x

p-value < 0.05

Abbreviations: aa, amino acid; *F* gene, fusion gene; FEL, fixed effects likelihood; HPIV1, Human parainfluenza virus type 1; HPIV3, Human parainfluenza virus type 3; IFEL, internal fixed effects likelihood; SLAC, single likelihood ancestor counting.

Table 2. Negative selection sites in the HPIV1 and HPIV3 *F* genes

Virus	SLAC	FEL	IFEL
HPIV1	13	46	11
HPIV3	74	124	69

p-value < 0.05

Abbreviations: F, fusion; FEL, fixed effects likelihood; HPIV1, human parainfluenza virus type 1; HPIV3, human parainfluenza virus type 3; IFEL, internal fixed effects likelihood; SLAC, single likelihood ancestor counting.

Table 3. Predicted linear B-cell epitopes in the HPIV1 and HPIV3 fusion proteins

Virus	Prototype strain (accession no.)	Position	Predicted epitopes
HPIV1	Washington/1964 (AF457102)	101-111	DTTVTNDNPQT
		341-350	YICPRDPTQL
		448-457	VGPAVSIRPV
		546-555	RNPYMGNNSN
HPIV3	Wash/47885/1957 (S82915)	57-66	IEDSNSCGDQ
		98-109	ESNENTDPRTKR
		337-346	SYICPSDPGF
		370-379	SDIVPRYAFV
		525-534	NRVDQNDKPY

Fig. 3. Position of amino acid substitution sites mapped to the predicted models of the fusion (F) protein in the Washington 1964 strain (the HPIV1 prototype strain).

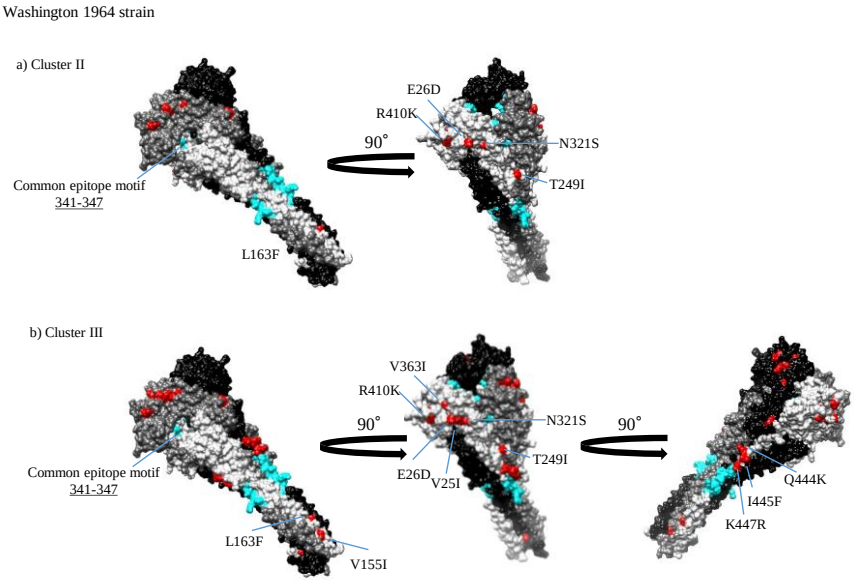


Fig. 4. Position of amino acid substitution sites mapped to the predicted models of the fusion (F) protein in the Wash/47885/57 strain (the HPIV3 prototype stain).

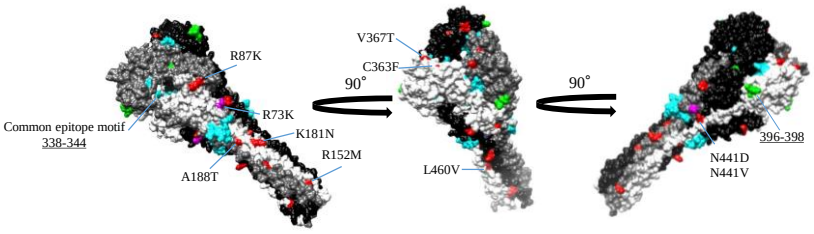
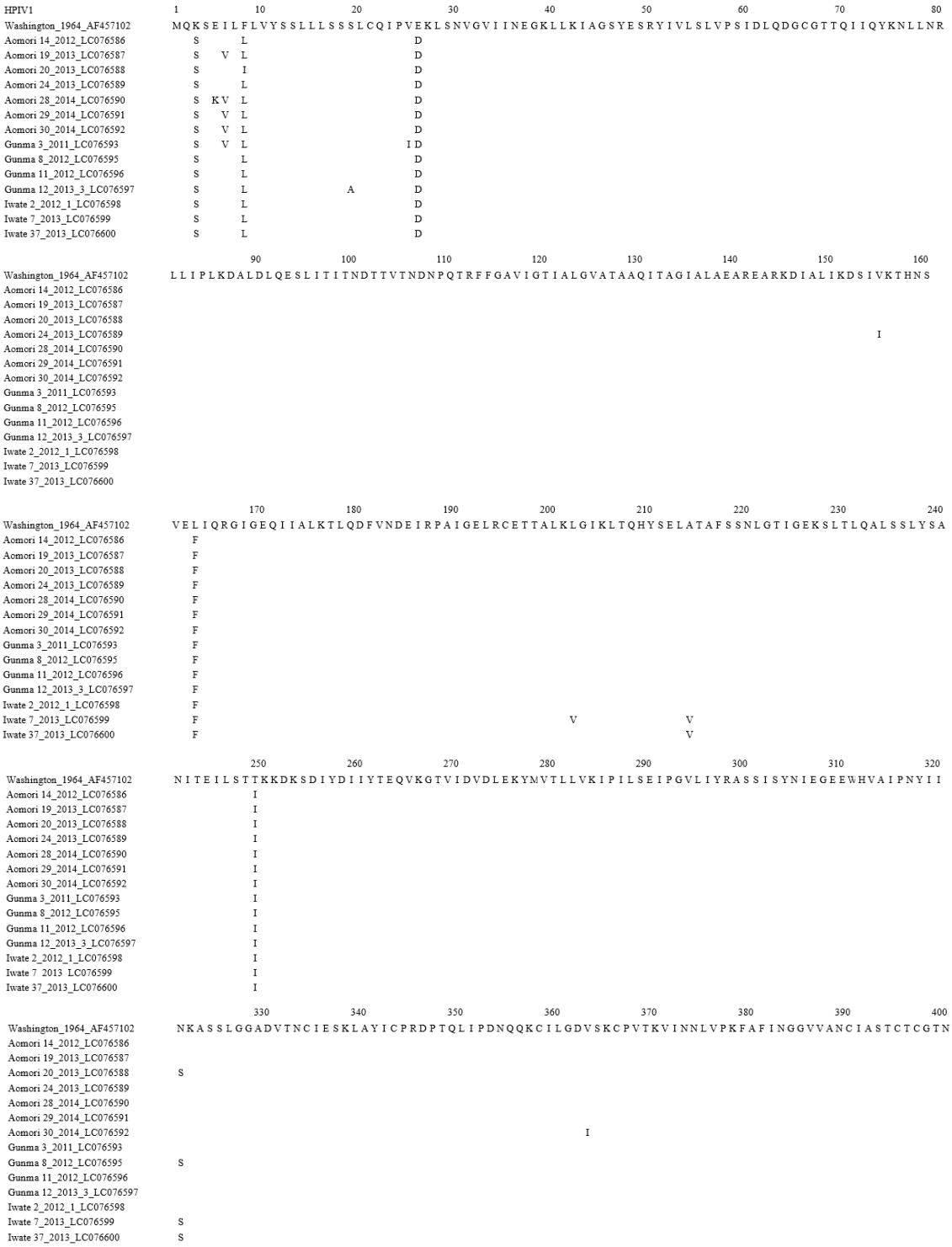


Table S2. Amino acid substituions in HPIV1



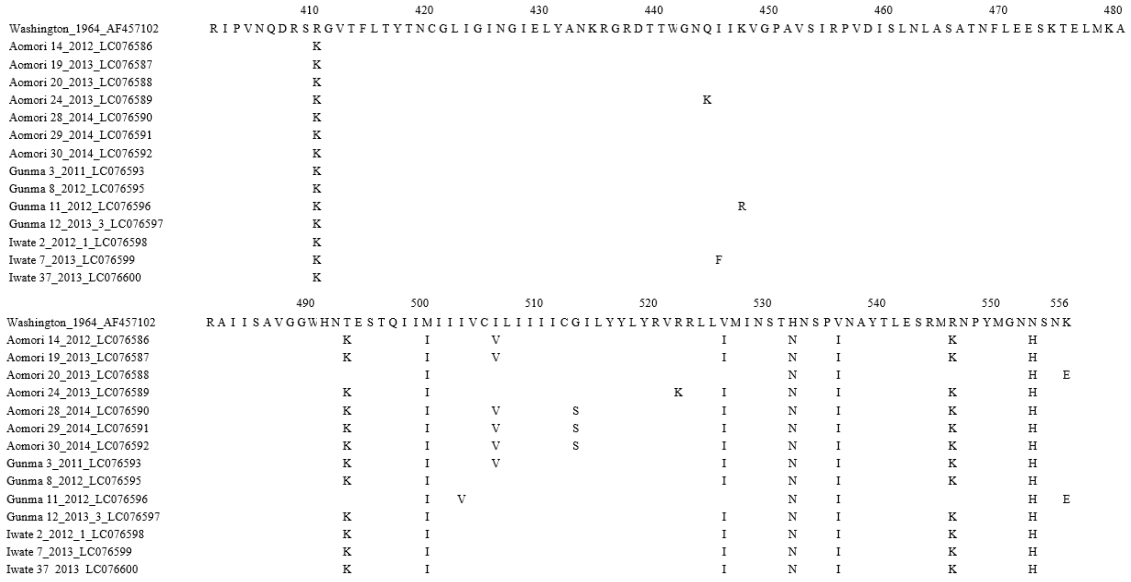

410 420 430 440 450 460 470 480
Washington_1964_AF457102 R I P V N Q D R S R G V T F L T Y T N C G L I G I N G I E L Y A N K R G R D T T W G N Q I I K V G P A V S I R P V D I S L N L A S A T N F L E E S K T E L M K A
Aomori 14_2012_LC076586 K
Aomori 19_2013_LC076587 K
Aomori 20_2013_LC076588 K
Aomori 24_2013_LC076589 K K
Aomori 28_2014_LC076590 K
Aomori 29_2014_LC076591 K
Aomori 30_2014_LC076592 K
Gunma 3_2011_LC076593 K
Gunma 8_2012_LC076595 K
Gunma 11_2012_LC076596 K R
Gunma 12_2013_3_LC076597 K
Iwate 2_2012_1_LC076598 K
Iwate 7_2013_LC076599 K F
Iwate 37_2013_LC076600 K
490 500 510 520 530 540 550 556
Washington_1964_AF457102 R A I I S A V G G W H N T E S T Q I I M I I I V C I L I I I I C G I L Y Y L Y R V R R L L V M I N S T H N S P V N A Y T L E S R M R N P Y M G N N S N K
Aomori 14_2012_LC076586 K I V I N I K H
Aomori 19_2013_LC076587 K I V I N I K H
Aomori 20_2013_LC076588 I N I H E
Aomori 24_2013_LC076589 K I K I N I K H
Aomori 28_2014_LC076590 K I V S I N I K H
Aomori 29_2014_LC076591 K I V S I N I K H
Aomori 30_2014_LC076592 K I V S I N I K H
Gunma 3_2011_LC076593 K I V I N I K H
Gunma 8_2012_LC076595 K I I N I K H
Gunma 11_2012_LC076596 I V N I H E
Gunma 12_2013_3_LC076597 K I I N I K H
Iwate 2_2012_1_LC076598 K I I N I K H
Iwate 7_2013_LC076599 K I I N I K H
Iwate 37 2013 LC076600 K I I N I K H

Table S3. Amino acid substituions in HPIV3

HPIV3	1									10										20										30										40										50										60										70										80
Wash_47885_1957_S82195	M	P	T	S	I	L	L	I	I	T	T	M	I	M	A	S	F	C	Q	I	D	I	T	K	L	Q	H	V	G	V	L	V	N	S	P	K	G	M	K	I	S	Q	N	F	E	T	R	Y	L	I	L	S	L	I	P	K	I	E	D	S	N	S	C	G	D	Q	Q	I	K	Q	Y	K	R	L	L	D	R	L	I	I
Aomori 1_2011_LC076601		L	I														H																																																																
Aomori 2_2011_1_LC076602		L	I														H																																																															
Aomori 3_2011_LC076603			I														H																																																															
Aomori 4_2011_LC076604		L	I		T												H																																																															
Aomori 5_2011_1_LC076605		L	I		T												H																																																															
Aomori 6_2011_LC076606			I														H																																																															
Aomori 7_2011_LC076607			I														H																																																															
Aomori 8_2012_4_LC076608		L	I				S										H																																																															
Aomori 10_2012_1_LC076609			I											T			H																																																														
Aomori 11_2012_LC076610		L	I				S										H																																																															
Aomori 18_2012_LC076611			I														H																																																															
Aomori 21_2013_3_LC076612			I														H																																																																
Aomori 22_2013_LC076613			I														H																																																																
Aomori 25_2014_LC076614		L	I														H																																																																
Aomori 27_2014_LC076615		L	I														H																																																																
Gunma 6_2012_LC076616		L	I														H																																																																
Iwate 3_2013_LC076617			I														H																																																																
Iwate 4_2013_LC076618			I														H																																																																
Iwate 5_2013_LC076619		L	A		T												H																																																																
Iwate 8_2013_LC076620			I							I							H																																																															
Iwate 10_2013_LC076621			I							I							H																																																															
Iwate 11_2013_7_LC076622			I														H																																																																
Iwate 12_2013_LC076623			I														H																																																																
Iwate 13_2013_LC078993			I														H																																																																
Iwate 16_2013_LC076624			I														H																																																																
Iwate 17_2013_LC076625			I														H																																																																
Iwate 18_2013_3_LC076626			I														H																																																																
Iwate 19_2013_LC076627		L	I				S										H																																																								K							
Iwate 22_2013_2_LC076628			I														H																																																																
Iwate 28_2013_2_LC076629		L	A		T												H																																																																
Iwate 32_2013_LC076630			I									I					H																																																															

										90										100										110										120										130										140										150										160
Wash_47885_1957_S82195	P	L	Y	D	G	L	R	L	Q	K	D	V	I	V	S	N	Q	E	S	N	E	N	T	D	P	R	T	K	R	F	F	G	G	V	I	G	T	I	A	L	G	V	A	T	S	A	Q	I	T	A	A	V	A	L	V	E	A	K	Q	A	R	S	D	I	E	K	L	K	E	A	I	R	D	T	N	K	A	V	Q	S
Aomori 1_2011_LC076601							K								T													E																																													M								
Aomori 2_2011_1_LC076602							K								T													E																																																						
Aomori 3_2011_LC076603															T													G																																																						
Aomori 4_2011_LC076604							K								T													E																																																						
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Discussion

In this study, we analyzed the *F* genes (full length) of both HPIV1 and HPIV3 among patients with ARIs in Eastern Japan between 2011 and 2015. The detection rate for both strains was about 3.5% (72 of the 2,069 patients with ARIs in this study). The phylogenetic trees indicated that the present HPIV1 and HPIV3 strains could be classified into two and one clusters, respectively, but that the dominant strains were in cluster III and C, respectively (Fig. 1a and 1b). The *p*-distance data also suggested that the genetic distances of the HPIV1 and HPIV3 strains were relatively low (mean *p*-distance < 0.03). Several positive selection sites were inferred in both of the present HPIV strains, although multiple negative selection sites were also inferred. Some epitopes of the F protein in the HPIV prototype strains were predicted, and a positive selection site was involved in an epitope of the HPIV3 F protein. Furthermore, some amino acid substitutions were estimated in the head and stalk of the F proteins for both HPIV1 and HPIV3. Our results indicated that the *F* gene of the prevalent HPIV1 and HPIV3 strains were relatively well conserved in these areas during the investigation period.

HPIV 3 and 1 were classified some lineages; for example, Mizuta et al [29, 30] have shown that HPIV3 isolated in Yamagata prefecture, Japan, could be classified into three lineages according to *HN* gene analysis [30]. They also reported that HPIV1 isolated in the same areas could be classified into two clusters according to the *HN* gene [29]. However, to the best of our knowledge, there are no reports regarding the genetic analysis of the *F* gene in HPIV1 and HPIV3 detected in Japan. In this study, we showed that HPIV1 and HPIV3 detected in the Eastern part of Japan could be classified into three and two lineages, respectively, by *F* gene analysis. Our results also indicated that both strains were well conserved. To compare the phylogenetics of the *F* genes of

HPIV1 and HPIV3 with those detected in Asia, America, Africa, and Europe, we tried to collect comprehensive data on the HPIV1 and HPIV3 *F* gene sequences from GenBank (full length). Both the strains in this study and the foreign strains were closely located in the phylogenetic trees. Furthermore, the genetic distances (*p*-distances) of these strains were relatively short. Thus, HPIV1 and HPIV3 *F* genes might have similar genetic properties in these strains, though we were unable to collect adequate numbers of *F* gene sequences for the virus strains. To better understand the genetic properties of the *F* genes in these strains, larger studies are needed.

Next, we inferred the positive and negative selection sites in the present strains. A few positive selection sites were inferred in the F proteins of both HPIV1 and HPIV3. The positive selection sites of the F protein in HPIV1 were located in the N-terminus of the protein (Lys5Glu, Gly5Glu, or Asp5Glu and Ile8Leu or Phe8Leu). Previous research has suggested that N-terminal amino acid residues 1–19 of the HPIV1 F protein act as the signaling peptide and that amino acid substitutions are common in these regions [24]. Thus, it is plausible that the amino acid substitutions in the present strains may cause alteration of the function of the F protein as a signaling peptide [18]. In contrast, the positive selection sites of the F protein in HPIV3 were located at amino acid residue 108 (Lys108Glu or Gly108Glu, and Glu108Lys or Gly108Lys), although this region consisted of a portion of the fusion peptide [24]. In recent years, it has been suggested that a mutation in the F protein may affect the growth rate and membrane fusion activity of HPIV [27, 28, 47]. Thus, these amino acid substitutions may reflect functional changes of the HPIV3 F protein, though we did not examine the molecular activities of the protein *in vitro*.

When viral antigen receives immune pressure by the host, the virus may try to escape recognition using a variety of evasion mechanisms [15]. Thus, it may be important to examine the relationships between the positive selection sites

and epitopes. Considering these circumstances, we predicted the linear epitopes in the HPIV F proteins as previously described [21]. As shown in Tables 1 and 3, a linear epitope was found to be involved in a positive selection site (aa 108) within the HPIV3 F protein. Moreover, this site may be highly conserved and essential for the fusion process. Thus, this site may not be associated with neutralizing sites. However, it remains unknown as to how such sites are involved in the function of the epitope and evasion of antibody detection initiated by the host defense mechanisms [36]. In addition, this site was incompatible with the liner epitopes and neutralization sites of mouse monoclonal antibodies. This may be due to differences in epitope recognition by the immune system between mice and humans [9]. Further studies regarding these relationships may be required.

To assess the relationships between amino acid substitutions and antibody reactivity against F proteins, we also modeled the F protein. This modeling showed that some amino acid substitutions could be predicted. Of these, R73K, which could be a neutralization-related amino acid, was detected in only one HPIV3 strain (LC76627) [5]. Additional surveillance is needed to elucidate the prevalence and clinical relevance of the mutation. In contrast, we could not determine the amino acids pertaining to neutralization-related sites in the HPIV1 F protein, since there is a lack of data pertaining to the neutralization sites of the F protein of HPIV1, similar to HPIV3. Overall, our data was based on relatively small numbers of each strain, making such assessment difficult.

In conclusion, we conducted a genetic analysis of the *F* genes of HPIV1 and HPIV3 from samples taken in Eastern Japan between 2011 and 2015. Despite this research it remains important to continue to accumulate additional data about both strains, and we recommend larger molecular epidemiology studies in the future. Indeed, although HPIV is a major cause of ARIs, there have been too few genetic studies with regard to the most common strains, HPIV1 and

HPIV3, and there is a need to remedy this situation in the future.

Acknowledgments

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要旨

2011 年から 2015 年にかけて、東日本における急性呼吸器感染症（ARI）患者から検出されたヒトパラインフルエンザウイルス 1 型（HPIV1,1668nt）および 3 型（HPIV3,1620nt）の融合タンパク質遺伝子（F 遺伝子）の全長を解析した。最尤法を用いて系統樹を構築し、遺伝的距離（p-distance）および positive selection site および negative selection sites を解析した。プロトタイプ株におけるタンパク質の線状エピトープも予測した。さらに、本株のタンパク質のアミノ酸置換をマッピングした。HPIV1 は 2 つのクラスター、HPIV3 は 1 つのクラスターに分類された。HPIV1 および HPIV3 F 遺伝子の p-距離値は<0.03 であった。HPIV1 は 2 つの positive selection sites を、HPIV3 は 1 つの positive selection sites を検出した。また、両方の株について、10 以上の negative selection sites を明らかにした。HPIV1 のプロトタイプ株は 4 つのエピトープ、HPIV3 のプロトタイプ株は 5 つのエピトープが予測された。HPIV3 で予測されたエピトープは positive selection site（aa108）に関与していた。さらに、LC76627 株におけるアミノ酸置換（R73K）は HPIV3 の 1 株のみで検出され（LC76627）、モノクローナル抗体による中和に対する耐性に影響を及ぼすことが考えられた。これらの結果は、調査期間中の東日本において、HPIV1 および HPIV3 の F 遺伝子が比較的よく保存されていることを示唆している。