A characteristic of polymorphic membrane protein F of *Chlamydia trachomatis* isolated from male urogenital tracts in Japan

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ABSTRACT

Although sexually transmitted disease due to *Chlamydia trachomatis* occurs similarly in both men and women, the female urogenital tract differs from that of males anatomically and physiologically, possibly leading to specific polymorphisms of the bacterial surface molecules. In the present study, we therefore characterized polymorphic features in a high-definition phylogenetic marker, polymorphic outer membrane protein (Pmp) F of *C. trachomatis* strains isolated from male urogenital tracts in Japan (Category: Japan-males, *n*=12), when compared with those isolated from female cervical ducts in Japan (Category: Japan-females, *n*=11), female cervical ducts in the other country (Category: Ref-females, *n*=12) or homosexual male rectums in the other country (Category: Ref-males, *n*=7), by general bioinformatics analysis tool with MAFFT software. As a result, phylogenetic reconstruction of the PmpF amino acid sequences showing three distinct clusters revealed that the Japan-males were limited into cluster 1 and 2, although there were only four clusters even though including an outgroup. Meanwhile, the phylogenetic distance values of PmpF passenger domain without hinge region, but not its full-length sequence, showed

that the Japan-males were more stable and displayed less diversity when compared with the other categories, supported by the sequence conservation features. Thus, PmpF passenger domain is a useful phylogenetic maker, and the phylogenic features indicate that *C. trachomatis* strains isolated from male urogenital tracts in Japan may be unique, suggesting an adaptation depending on selective pressure, such as the presence or absence of microbial flora, furthermore possibly connecting to sexual differentiation.

1. Introduction

Obligate intracellular bacterium *Chlamydia trachomatis* is a causative agent of sexually transmitted diseases, leading to serious complications involving infertility (e.g., in females: ductal obstruction, pelvic inflammatory disease, tubal occlusion; in males: testicular atrophy, epididymitis, orchitis)[1-6]. Chlamydial infection occurs similarly in both men and women. However, the female vagina with microbial flora differs from that of male urogenital tract without the flora anatomically and physiologically [7], suggesting that *C. trachomatis* strains would be subjected to different selective pressures, possibly leading to specific polymorphisms of the bacterial surface molecules depending on sexual differentiation. To date, most studies of *C. trachomatis* bacterial traits have focused on strains isolated from women, whereas the study of bacteria from the male urogenital tract has been hindered by difficulties in the isolation of organisms. Meanwhile, accumulating evidences have revealed a high-definition phylogenetic marker, polymorphic membrane protein (Pmp), which diversifies into nine Pmp families (A–I) in *C. trachomatis* [8-13]. Proteins within the Pmp family generally exhibit the properties of type V secretion systems,

which include: an N-terminal signal sequence associated with transport across the plasma membrane, a C-terminal β-barrel domain embedded into the outer membrane, and a passenger domain exposed to the surface of outer membrane across the channel formed by the β-barrel structure, connected through a hinge region (See Fig. 4, top scheme) [14]. Thus, the surface exposure of the PmpF molecule indicates that it could be strongly subjected to host-selective pressures such as microbial flora [15]. In fact, phylogenetic studies of *pmp* genes found that among them *pmpF* exhibited the greatest genetic diversity, both at the nucleotide and amino acid level, among *C. trachomatis* genital strains [13, 16].

In the present study, we therefore evaluated polymorphisms in PmpF of *C. trachomatis* isolated from male urogenital tracts in Japan, when compared with those isolated from female cervical ducts in Japan or homosexual male rectums in the other countries, by a general bioinformatics analysis tool with MAFFT software.

2. Materials and methods

2.1 Bacterial strains and PmpF amino acid sequence information

Forty-two PmpF amino acid sequences of *C. trachomatis* strains were used for the study; these included strains isolated from male urogenital tracts in Japan (Category: Japan-males, *n*=12), female cervical ducts in Japan (Category: Japan-females, *n*=11) and reference (Ref) strains isolated from either homosexual male rectums or female cervical ducts in other countries (Category: Ref-males, *n*=7; Ref-females, *n*=12). Regarding this, we selected the Japan-female strains with *ompA* genotypes coincident with the Japan-male strains. At the beginning, nineteen strains of *C. trachomatis* isolated from male urogenital tracts in Japan were purchased from the Japanese surveillance committee, consisting of the Japanese Society of Chemotherapy, the Japanese Associations for Infectious Diseases and the Japanese Society for Clinical Microbiology (Tokyo, Japan). Meanwhile, twelve of these strains only successfully propagated in HEp-2 cells (Strain numbers: 54, 404, 414, 419, 440, 441, 519, 520, 626, 627, 664, and 687) were used for the study (category: Japan-males) (See below) [17]. As a homosexual male reference group, seven of the male strains obtained from rectums in the current study (Strain numbers: 70, 150, 2923, 6276, 9301,

9768, 11023, 11074, and 11222) that has been reported previously [18], were also used for the study. Furthermore, as female strain groups, eleven of the female strains used in the present study were previously isolated from Sapporo, Japan (Strain designations: nv266, nv283, nv287, nv307, nv381, nv399, nv441, nv417, nv437, nv443, and nv448), as previously reported [19] (Category: Japan-females); the other 12 female strains (Strain designations: CS-431/04, I-174, I-149, I-24, CS-190/96, I-139, CS-121/96, CS-490/95, S-91, 537C-05, S-141, and CS-500-96) had also been previously reported [11, 16] (Category: Ref-females). The origins and accession numbers of all of the strains are summarized in Table 1. Fig. 1 also shows the difference of geographical locations between the Japan-male strains (isolation places spread in whole Japan) and the Japan-female strains (isolation places limited in Sapporo, Japan).

2.2. *Bacterial growth condition*

As mentioned above, the strains were propagated in HEp-2 cells, cultured with DMEM supplemented with 20% FCS and antibiotics (10 μg/ml gentamicin, 10 μg/ml

vancomycin and 1 μg/ml amphotericin B) (Sigma, St. Louis, MO, USA) [17]. In brief, The infected cells were harvested at 72 h after infection, and stored at −80°C. The cell suspensions were disrupted by freezing–thawing and centrifugation at 400 × g to remove cell debris, and then the bacteria collected by centrifugation at 1,000 × g were resuspended in sucrose–phosphate–glutamic acid buffer (0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4 and 5 mM L-glutamic acid). The bacteria were stored at −80°C until use.

2.3. Phylogenetic determination, sequence identity and conservation analysis

For determining *ompA* and *pmpF* sequences, bacterial genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. Extracted DNA was amplified using High-Fidelity Phusion DNA polymerase (Thermo Fisher Scientific Inc., San Jose, CA, USA) and specific primer sets for *ompA* (for genotyping) and *pmpF*, according to previous studies [13, 19]. The primer sets are described in the supplementary material (Table S1). The amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Amplified products were excised from the gel using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) according to the manufacture's protocol, and sequenced by Macrogen (Macrogen, Seoul, South Korea). Obtained all nucleotide sequences were deposited to DNA Data Bank of Japan (See Table 1). Phylogenetic analysis, along with determination of pairwise distances, sequence identity analysis and conservation analysis were conducted using MAFFT version 7 software (http://mafft.cbrc.jp/alignment/software/) [20]. For phylogenetic analysis, four *C. trachomatis* L1-3 strains (accession numbers: AY887659, AY887660, AY887661, AY887662) were used as an out-group.

2.4. Prediction of PmpF domain structure

The PmpF domain structure of *C. trachomatis* strain UW-3 (genotype D) (accession number: AY887648) was also predicted using SMART software (http://smart.embl-heidelberg.de/) [21].

2.5. Statistical analysis

The average pairwise distance values for each of the categories (Japan-males, Ref-males, Japan-females and Ref-females) were defined as the phylogenetic distance values and were compared between these categories. Variance of the data between the categories was assessed by one-way analysis of variance and multiple regression analysis (one-way ANOVA). Comparison of the data between the categories was assessed by Bonferroni's post-hoc multiple comparison test. A P value <0.05 was considered significant.

3. Results

Phylogenetic reconstruction of the PmpF amino acid sequences revealed three distinct clusters with no out-group (Fig. 2). Interestingly, all clusters (clusters 1-3) contained Ref-males, Japan-females and Ref-females, but did not Japan-males; all of the Japan-males were seen in phylogenically distinct two branches (cluster 1 and 2), although

there were only four clusters even though including an outgroup.

To investigate this further, phylogenetic distance values were determined and compared between the categories. At first, full-length amino acid sequences of PmpFs were compared. However, as a result, there was no significant difference of the phylogenetic distance values among the categories (Average±standard deviation: Japan-males, 0.147±0.178; Ref-males, 0.192±0.204; Japan-females, 0.171±0.194; Ref-females, 0.166±0.200) (Variance: p=0.798, one-way ANOVA; Comparison: See Fig. 3A, Bonferroni) (Fig. 3A). Meanwhile, it is likely that such total average distance values in the full length PmpFs could not show the presence of diversity accurately because of uneven distribution of diversity in the sequences. In fact, as mentioned above, PmpF type V secretion systems consist of two distinct structures including a C-terminal β-barrel domain embedded into the outer membrane and a passenger domain exposed to the surface of outer membrane across the channel formed by the β-barrel structure, suggesting the surface exposed portion across outer membrane subjected to more host-selective pressures [14]. Accordingly, passenger domain amino acid sequences of PmpFs (approximately 670 amino

acid residues, excluded "hinge region" with similarly variable sequences between males sand females), possibly with more diverse, were compared as the next step. As a result, it is expected that the Japan-male values was the lowest among the categories and more importantly that there was a statistical significant difference of the distant values between the Japan-males and the other categories (Average±standard deviation: Japan-males, 0.0002±0.0005; Ref-males, 0.0219±0.0213; Japan-females, 0.0198±0.0227; Ref-females, 0.0152±0.0211) (Variance: p=2.286E-09, one-way ANOVA; Comparison: See Fig. 3B, Bonferroni) (Fig. 3B).

Finally, sequence alignments constructed using MAFFT software were compared between the categories, and the levels of sequence identity and the conservation of specific moieties on the PmpF molecule were analyzed. As a result, all the categories showed similar levels of heterogeneity on the hinge sequence (between the passenger domain and the β-barrel domain) and the β-barrel domain with minimal substitutions (Fig. 4) (also see supplemental data for high-resolution images, Fig. S1). Meanwhile, in contrast to the other categories, the passenger domain sequences without hinge region from 670 to 762 amino

acid residues in the Japan-males were completely conserved, except for a specific amino acid substitution identified in the signal sequence [amino acid at position 3 (K to E); 626 strain only] (Fig. 4, Japan_males) (also see supplemental data for high-resolution images, Fig. S1).

Thus, the findings indicated that the PmpF amino acid sequences of Japan-male strains used for this study are very unique with less diverse, suggesting that selective pressure on *C. trachomatis* in the male urogenital tract may be less than in the female cervical ducts.

4. Discussion

It has previously been established that the PmpF C-terminal β-barrel domain embedded in the outer membrane shows clear evolutionary conservation, supporting the function of this domain as a critical channel [13, 14]. In contrast to the β-barrel domain, the PmpF passenger domains have been shown to undergone amino acid changes throughout

evolution because of surface localization [13-15]. Thus, polymorphism of the PmpF passage domain, which is exposed to strong host-selective pressures, has been thought to be a candidate high-definition phylogenetic marker [8-13]. The Japan-female strains isolated from cervical ducts with microbial flora were very diverse in agreement with the Ref-females as well as previous findings [12, 13]. Correspondingly, the homosexual male PmpFs of *C. trachomatis* strains isolated from rectum with microbial flora were similarly in diverse as well as female PmpFs, supporting our hypothesis. Meanwhile, this is the first study to analyze Japanese-male PmpFs, and the findings were contradictory to the female and homosexual male data, suggesting that the *C. trachomatis* strains isolated from male urogenital tract may be very unique.

At this time, the rational reason why only the Japan-male PmpFs is more stable/less variable remains unknown. Meanwhile, we speculate that distinct selective pressures, such as anatomical feature either with or without normal microbial flora, may target the PmpF molecule, possibly connecting sexual differentiation. In fact, the previous evidences showing that in contrast to ocular strains vaginal microbial flora could allow *C. trachomatis*

urogenital strains to escape IFN-gamma-mediated eradication and establish persistent infection [18, 22], is supporting our speculation. In addition, since it has been reported that *C. trachomatis* appears to reveal a slow *in vitro* adaptation to cells [23], effect of short-term laboratory propagation on *C. trachomatis* genomic mutations is thought to be minimal.

It has generally been believed that chlamydial sexually transmitted disease results from reciprocal bacterial transmission between partners. Meanwhile, although this research was a small scale study with limited data, our data propose a possible feature that some *C. trachomatis* strains inhabited in urogenital tract of male with less selective pressure, such as lacking normal flora, may be nascent, however after transmitted to female urogenital tract becoming more diverse through adaptation to vagina with microbial flora. In addition, the fact that in contrast to the Japan-female strains the Japan-male strains used for this study were isolated from geographically distinct places in Japan, also support our hypothesis. Meanwhile, since it cannot deny the effect of the presence of commensal bacteria or co-infected pathogens on *C. trachomatis* genetic changes, further study should be needed to clarify these possibilities.

In conclusion, our study for the first time reported a comparison between PmpF sequences derived from *C. trachomatis*-infected males and females, showing a novel finding that the male PmpF in Japan is more stable with less diversity. Our findings indicate that PmpF passenger domain is a useful phylogenetic marker with high resolution to easily define *C. trachomatis* strain diversities or their evolution, and more importantly suggest that *C. trachomatis* strains may adapt to urogenital tract with less diversity because of lacking of microbial flora, possibly connecting sexual differentiation. Further, large-scale studies of *C. trachomatis* strains isolated from male urogenital tracts with whole genomic analysis are now required to investigate these host-specific adaptions in more detail and therefore better understand chlamydial pathogenesis.

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Conflict of interests

The authors declare that no competing interests exist.

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Figure legends

Fig. 1. Location map of *C. trachomatis* strains isolated from males (closed circular) and females (open circular) in Japan.

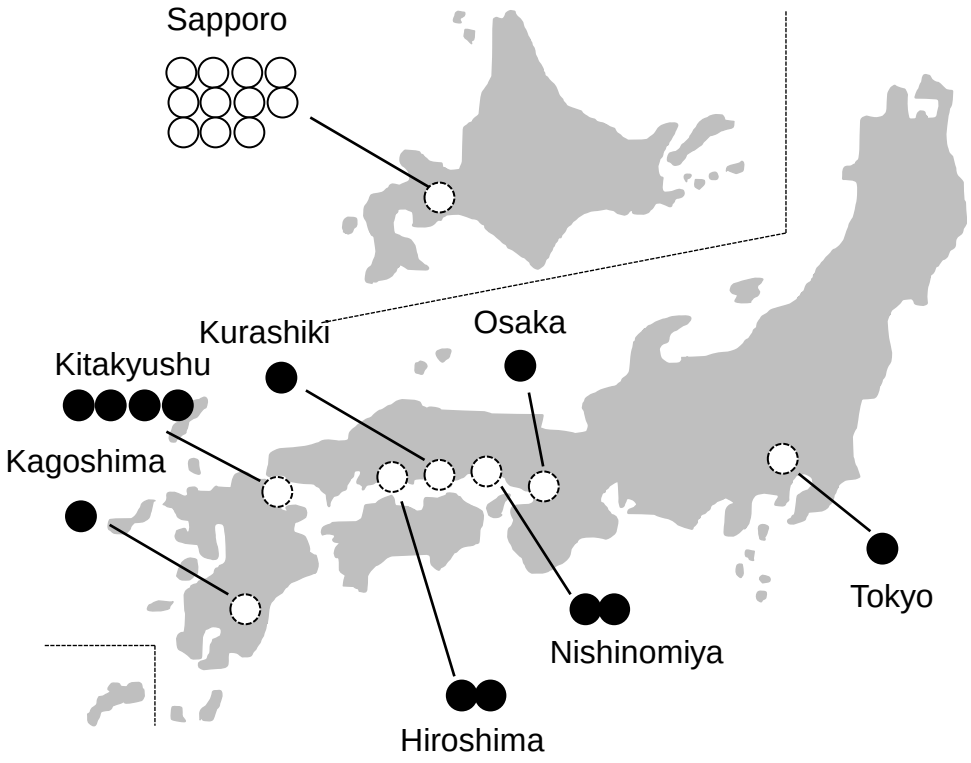
Fig. 2. Phylogenetic tree of the PmpF amino acid sequences showing PmpF diversity. The tree was constructed using MAFFT software with neighbor-joining tree topologies (See the text) [20]. The PmpF sequences of 24 distinct bacterial strains are included [Japan-males (male: M), *n*=12; Ref-males, *n*=7; Japan-females (female: F), *n*=11; Ref-females, *n*=11]. Closed circles, Japan-males. Closed triangles, Ref-males. Open circles, Japan-females. Open triangles, Ref-females. Letters in parentheses represent *ompA* genotypes. Letters and numbers in parentheses represent accession numbers. Also, see Table 1.

Fig. 3. Comparison of the phylogenic distance values of the PmpF of amino acid sequences, full length (A) and 670aa-passenger domain (B). The average pairwise distance values for

each of the categories were defined as phylogenic distance values and were compared between the categories. Values into boxes show statistical analysis values. Refer to the text: Phylogenetic determination and statistical analysis. *$p<0.01$, statistical significance.

Fig. 4. Assessment of sequence identity and conservation moieties within the PmpF amino acid sequences. Alignment construction, sequence identity and conservation analysis were performed using MAFFT software [20]. The PmpF domain structure of *C. trachomatis* strain UW-3 (genotype D) was predicted using SMART software (http://smart.embl-heidelberg.de/) [22] (Top scheme). Refer to the text: Phylogenetic determination and statistical analysis. Vertical lines into sequence boxes (*) show less conservation with low % identity.

Fig. 1


Sapporo
Kurashiki
Osaka
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Kagoshima
Tokyo
Nishinomiya
Hiroshima

Fig. 2

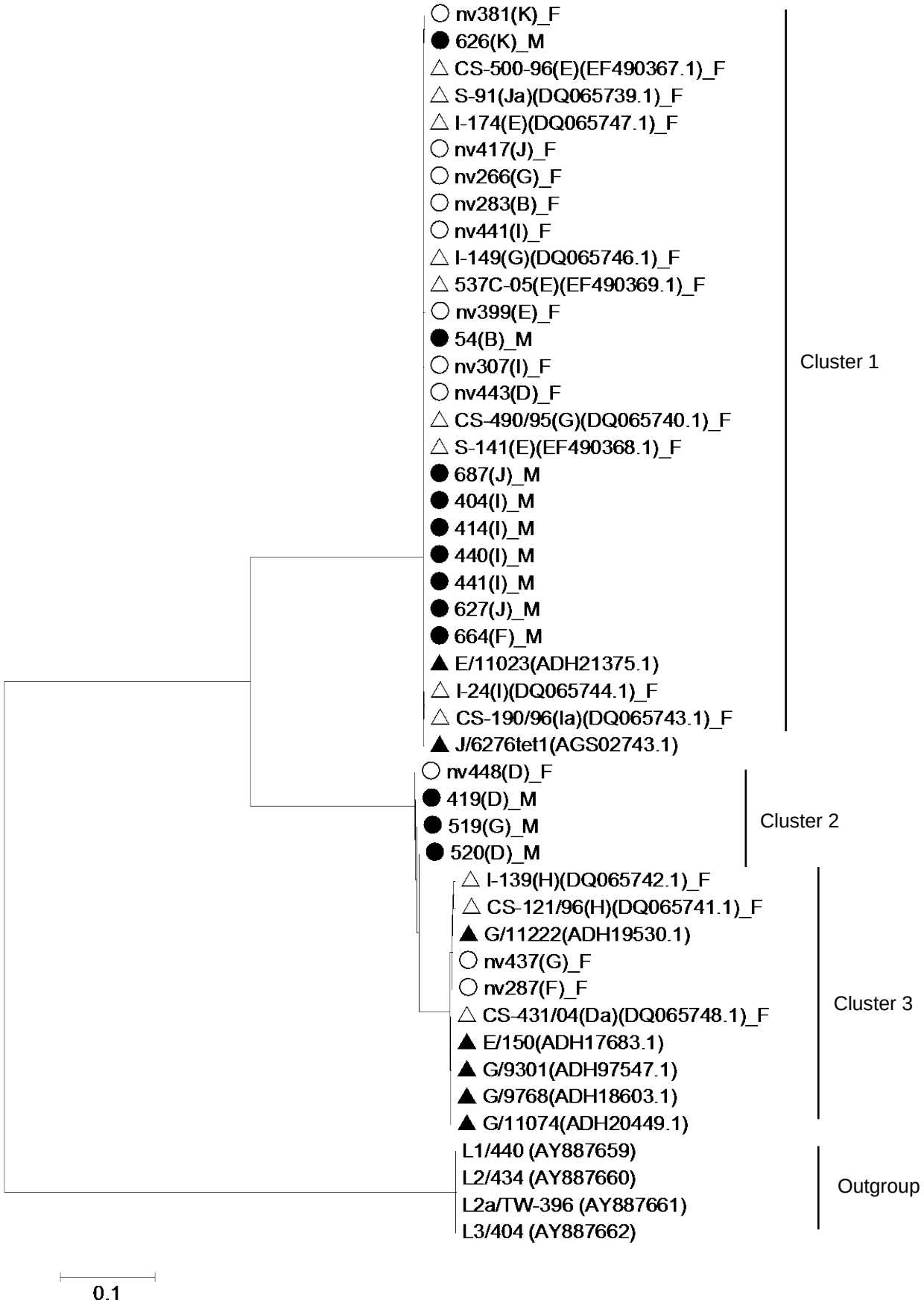
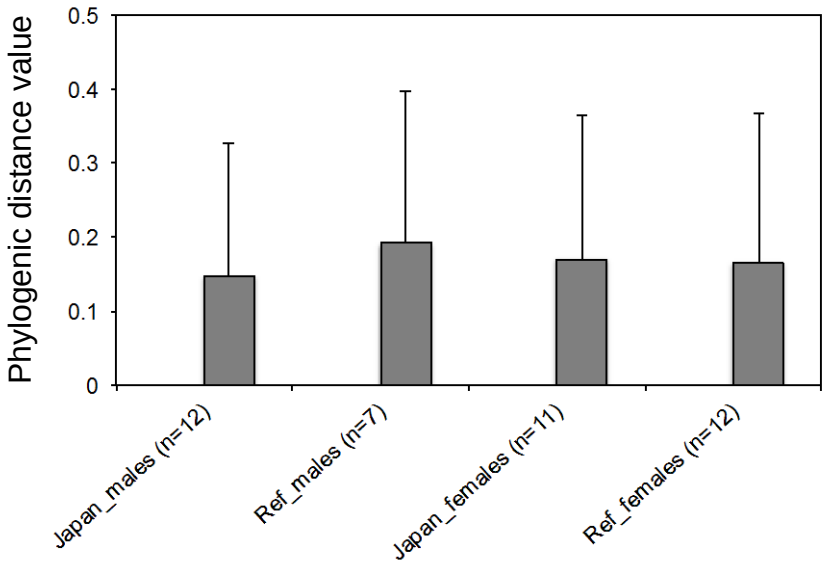

nv381(K)_F
626(K)_M
CS-500-96(E)(EF490367.1)_F
S-91(Ja)(DQ065739.1)_F
I-174(E)(DQ065747.1)_F
nv417(J)_F
nv266(G)_F
nv283(B)_F
nv441(I)_F
I-149(G)(DQ065746.1)_F
537C-05(E)(EF490369.1)_F
nv399(E)_F
54(B)_M
nv307(I)_F
nv443(D)_F
CS-490/95(G)(DQ065740.1)_F
S-141(E)(EF490368.1)_F
687(J)_M
404(I)_M
414(I)_M
440(I)_M
441(I)_M
627(J)_M
664(F)_M
E/11023(ADH21375.1)
I-24(I)(DQ065744.1)_F
CS-190/96(Ia)(DQ065743.1)_F
J/6276tet1(AGS02743.1)
Cluster 1
nv448(D)_F
419(D)_M
519(G)_M
520(D)_M
Cluster 2
I-139(H)(DQ065742.1)_F
CS-121/96(H)(DQ065741.1)_F
G/11222(ADH19530.1)
nv437(G)_F
nv287(F)_F
CS-431/04(Da)(DQ065748.1)_F
E/150(ADH17683.1)
G/9301(ADH97547.1)
G/9768(ADH18603.1)
G/11074(ADH20449.1)
Cluster 3
L1/440 (AY887659)
L2/434 (AY887660)
L2a/TW-396 (AY887661)
L3/404 (AY887662)
Outgroup
0.1

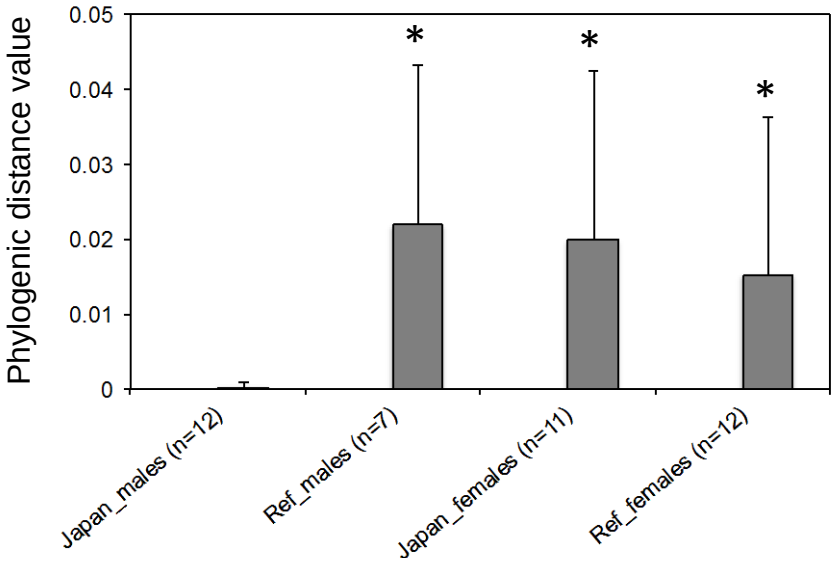
Fig. 3

A



	Japan_males	Ref_males	Japan_females	Ref_females
Japan_males (n=12)		1	1	1
Ref_males (n=7)			1	1
Japan_females (n=11)				1
Ref_females (n=12)				

B



	Japan_males	Ref_males	Japan_females	Ref_females
Japn_males (n=12)		0 *	0 *	0 *
Ref_males (n=7)			0.904	1
Japn_females (n=11)				1
Ref_females (n=12)				

Fig.4

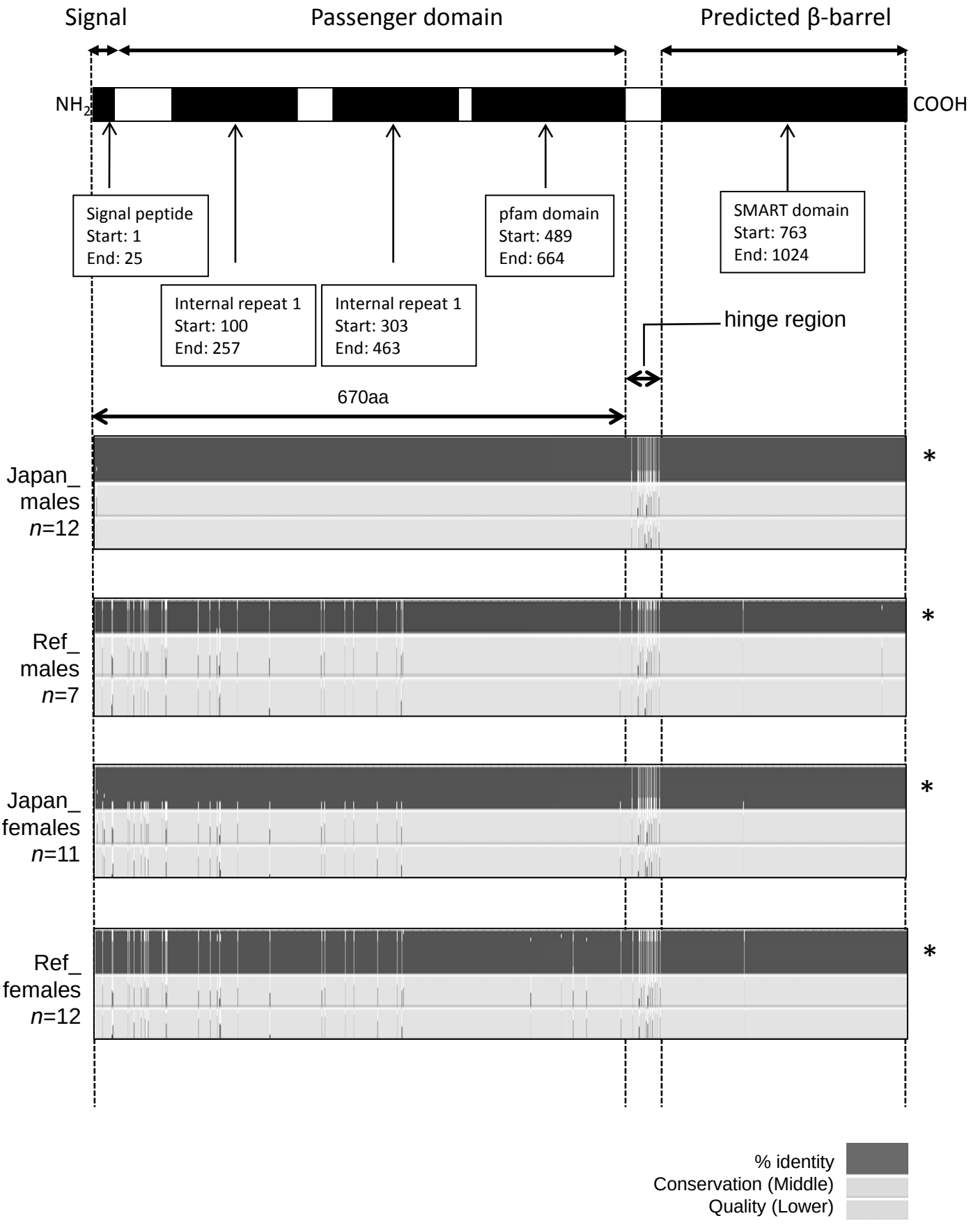
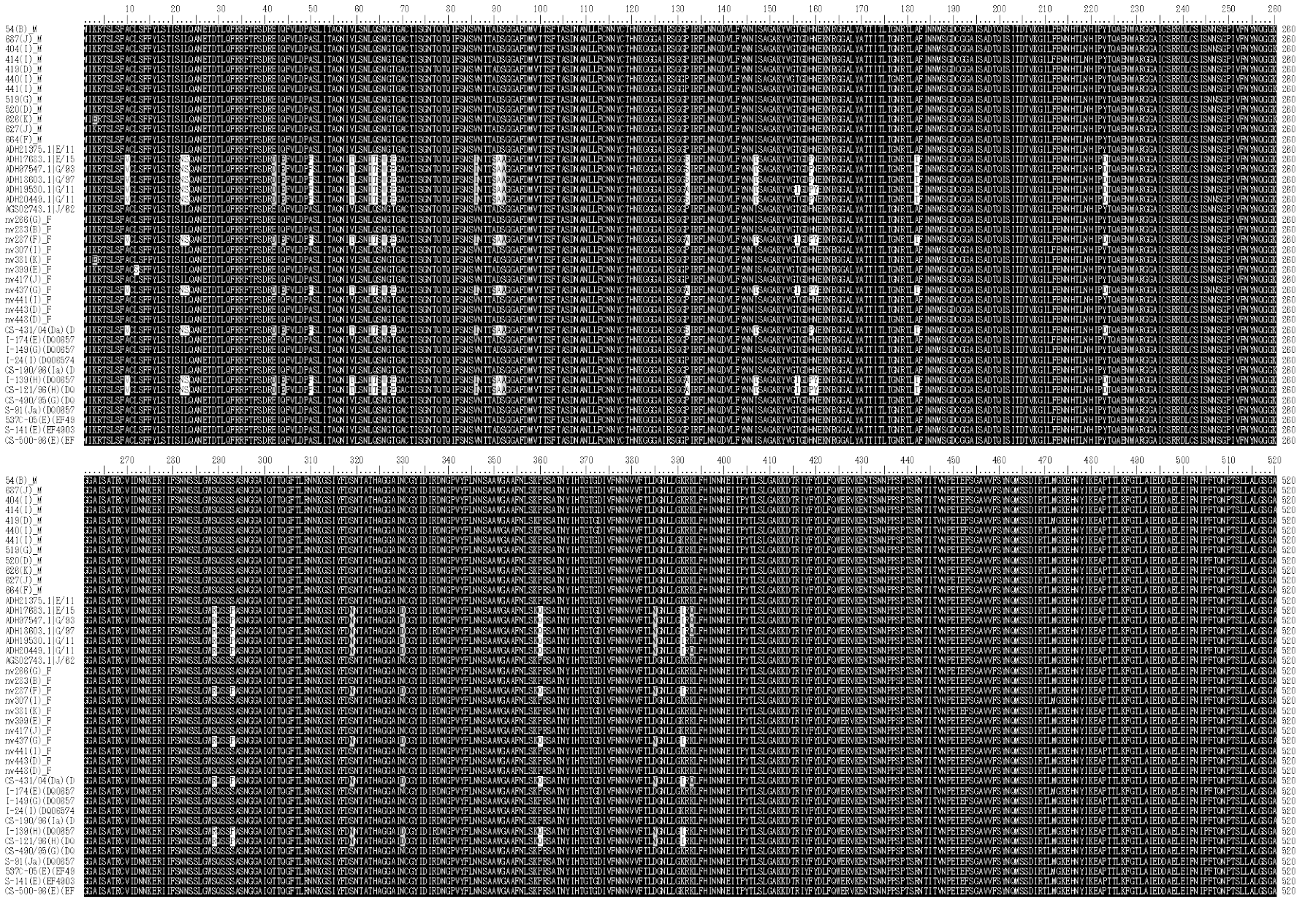

Signal
Passenger domain
Predicted β-barrel
NH2
COOH
Signal peptide
Start: 1
End: 25
Internal repeat 1
Start: 100
End: 257
Internal repeat 1
Start: 303
End: 463
pfam domain
Start: 489
End: 664
SMART domain
Start: 763
End: 1024
hinge region
670aa
Japan_ males n=12
Ref_ males n=7
Japan_ females n=11
Ref_ females n=12
*
% identity
Conservation (Middle)
Quality (Lower)

Fig. S1: high-resolution images showing PmpF amino acid sequence alignment



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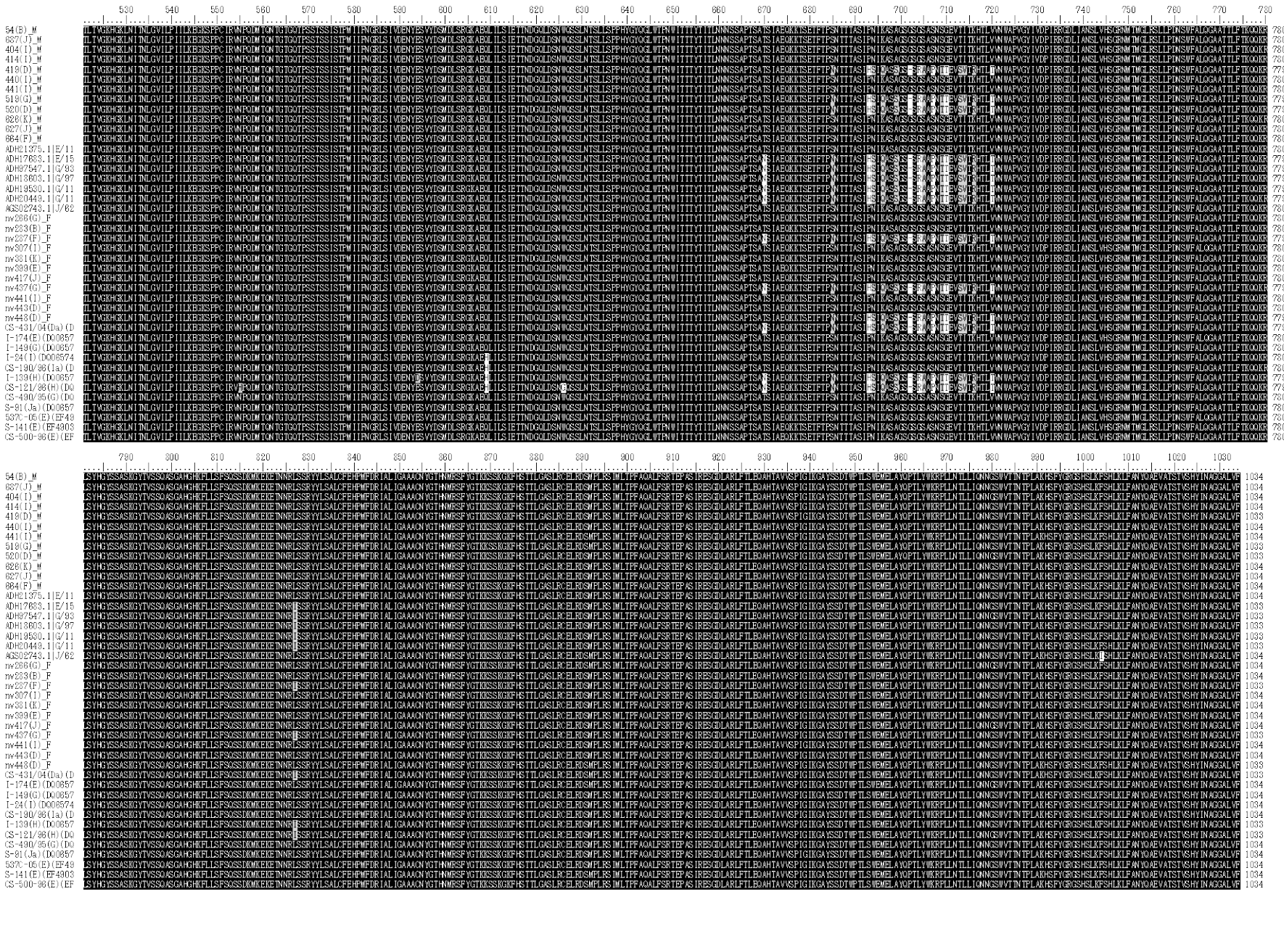

Table 1 Origins of *C. trachomatis* strians and *pmpF* accession numbers

Category	Strain name	Origen (Sex)	Isolated from	*ompA* genotype (accession number)	*pmpF* accession number	Notes	References
Japan-malse	404	Male	Urethra	I (LC031847)	LC031825	This study	
(*n* =12)	414	Male	Urethra	I (LC031848)	LC031826	This study	
	419	Male	Urethra	D (LC031849)	LC031827	This study	
	440	Male	Urethra	I (LC031850)	LC031828	This study	
	441	Male	Urethra	I (LC031851)	LC031829	This study	
	519	Male	Urethra	G (LC031852)	LC031830	This study	
	520	Male	Urethra	D (LC031853)	LC031831	This study	
	54	Male	Urethra	B (LC031846)	LC031823	This study	
	626	Male	Urethra	K (LC031854)	LC031832	This study	
	627	Male	Urethra	J (LC031855)	LC031833	This study	
	664	Male	Urethra	F (LC031856)	LC031834	This study	
	687	Male	Urethra	J (LC031857)	LC031824	This study	
Reference-malse	11023	Male	rectum	E (See reference)	ADH21375.1	Other study	[17]
(*n* =7)	150	Male	rectum	E (See reference)	ADH17683.1	Other study	[17]
	9301	Male	rectum	G (See reference)	ADH97547.1	Other study	[17]
	9768	Male	rectum	G (See reference)	ADH18603.1	Other study	[17]
	11222	Male	rectum	G (See reference)	ADH19530.1	Other study	[17]
	11074	Male	rectum	G (See reference)	ADH20449.1	Other study	[17]
	6276	Male	rectum	J (See reference)	AGS02743.1	Other study	[17]
Japan-females	nv266	Female	cervix	G (AB695149)	LC031835	This study (isolated previously)	[18]
(*n* =11)	nv283	Female	cervix	B (AB695151)	LC031836	This study (isolated previously)	[18]
	nv287	Female	cervix	F (AB695152)	LC031837	This study (isolated previously)	[18]
	nv307	Female	cervix	I (AB695154)	LC031838	This study (isolated previously)	[18]
	nv381	Female	cervix	K (AB695161)	LC031839	This study (isolated previously)	[18]
	nv399	Female	cervix	E (AB695162)	LC031840	This study (isolated previously)	[18]
	nv417	Female	cervix	J (AB695165)	LC031841	This study (isolated previously)	[18]
	nv437	Female	cervix	G (AB695170)	LC031842	This study (isolated previously)	[18]
	nv441	Female	cervix	I (AB695171)	LC031843	This study (isolated previously)	[18]
	nv443	Female	cervix	D (AB695172)	LC031844	This study (isolated previously)	[18]
	nv448	Female	cervix	D (AB695173)	LC031845	This study (isolated previously)	[18]
Reference-females	CS-431/04	Female	Urogenital	Da (See reference)	DQ065748.1	Other study	[13]
(*n* =12)	I-174	Female	Urogenital	E (See reference)	DQ065747.1	Other study	[13]
	I-149	Female	Urogenital	G (See reference)	DQ065746.1	Other study	[13]
	I-24	Female	Urogenital	I (See reference)	DQ065744.1	Other study	[13]
	CS-190/96	Female	Urogenital	Ia (See reference)	DQ065743.1	Other study	[13]
	I-139	Female	Urogenital	H (See reference)	DQ065742.1	Other study	[13]
	CS-121/96	Female	Urogenital	H (See reference)	DQ065741.1	Other study	[13]
	CS-490/95	Female	Urogenital	G (See reference)	DQ065740.1	Other study	[13]
	S-91	Female	Urogenital	Ja (See reference)	DQ065739.1	Other study	[13]
	537C-05	Female	Urogenital	E (See reference)	EF490369.1	Other study	[11]
	S-141	Female	Urogenital	E (See reference)	EF490368.1	Other study	[11]
	CS-500-96	Female	Urogenital	E (See reference)	EF490367.1	Other study	[11]

Table S1. Primers used in this study

Gene	Primer	Sequence	Gene location	Amplicon size (bp)	Reference
For *ompA* genotyping					
	P1	5'-ATGAAAAAACTCTTGAAATCGG-3'	1–22 (For full length amplification and seuqencing)	1,124	[18]
	OMP2	5'-ACTGTAACTGCGTATTTGTCTG-3'	1,124–1,103 (For full length amplification and sequencing)		[18]
For *pmpF* sequencing					
	PmpF-2	5'-CTTATCTTCAGCGCATTCGTCCTTC-3'	(−)91–(−)67 (For full length amplification and sequencing)	3,664	[13]
	PmpF-1	5'-GAATGGCTCCGCCTTCTCTTATTTT-3'	(+)468–(+)444 (For full length amplification and sequencing)		[13]
	F-7	5'-GCATTCGTCCTTCTCTCTTT-3'	(−)80–(−)60 (For sequencing)		[13]
	F-4	5'-TGACAGGGAATCGAACTCT-3'	513–543 (For sequencing)		[13]
	F-6	5'-AAATTATATCCATACAGGGA-3'	1,092–1,112 (For sequencing)		[13]
	F-8	5'-TTCACTAGTTTTTTTCTGCT-3'	2,041–2,021 (For sequencing)		[13]
	F-5	5'-GAGTGAAATTTCCCTTTAGA-3'	2,630–2,610 (For sequencing)		[13]
	F-3	5'-ATTCTAGAAGGAACCTCTCT-3'	(+)76–(+)56 (For sequencing)		[13]