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Genetic diversity of *Legionella pneumophila* inferred from *rpoB* and *dotA* sequences

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ABSTRACT

This study characterised the population structure of *Legionella pneumophila* by comparing the *rpoB* (300-bp) and *dotA* (360-bp) sequences of 267 isolates (18 reference strains, 149 Korean isolates and 100 Japanese isolates). In addition to the six clonal subgroups established previously, four subgroups, P-V to P-VIII, were identified. Subgroupings based on *rpoB* and *dotA* sequences were found to correlate with the source of the isolates, and this data may be useful for future epidemiological studies. Fourteen (five Korean and nine Japanese) isolates showed incongruent subgroupings in the *rpoB* and *dotA* trees, suggesting that genetic exchange among subgroups, and even among subspecies, may occur frequently in nature.

Keywords *dotA*, *Legionella pneumophila*, population structure, *rpoB*, sequence analysis, subgroup

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INTRODUCTION

Legionella pneumophila is a Gram-negative facultative intracellular pathogen that is responsible for both Legionnaires' disease and Pontiac fever [1]. It was first described in 1977, following an outbreak of pneumonia in Philadelphia during an American Legion Convention in 1976 [2]. *L. pneumophila* is distributed ubiquitously in natural and artificial water systems, and is known to preferentially colonise protozoan host cells and human alveolar macrophages [3]. Although more than two decades have elapsed since the first serious outbreak, Legionnaires' disease continues to attract public attention. The disease accounts for 4–20% of cases of community-acquired pneumonia, and is one of the principal causes of pneumonia requiring hospitalisation [4]. Recently, several outbreaks of *L. pneumophila*-associated pneumonia or Pontiac fever have been reported in Europe and North America [5–11]. Japan has also experienced several outbreaks since 1980. More than 300 individuals were infected with *L. pneumophila* in an outbreak at a Japanese hot spring resort in early August 2002, and eight of the infected individuals eventually died of the disease [12]. However, no epidemic outbreaks of legionellosis have occurred in Korea since an outbreak of Pontiac fever in 1984.

Sequence-based typing methods, including multilocus sequence typing, have been shown to be more effective than serotyping in epidemiological studies [13]. In general, sequence-based population studies have usually compared five to nine genes [14], as is the case with multilocus sequence typing. In this respect, if a method involving the use of a few genes was sufficiently accessible, relatively straightforward, not prohibitively

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expensive, and possessed sufficient resolving power to discriminate adequately between strains, it might prove to be a useful typing method.

The *rpoB* gene encodes the β-subunit of RNA polymerase, whereas *dotA* is hyper-variable, and encodes an integral cytoplasmic membrane protein (DotA). In a previous evaluation [15], *rpoB* and *dotA* were shown to be suitable targets for *L. pneumophila* population studies. Six clonal populations were identified among 79 Korean isolates collected between 1985 and 2000 (three isolates from the lung tissues of three sporadic clinical cases, and 76 isolates from different air-conditioning cooling tower water systems); four of these were shown to comprise *L. pneumophila* subsp. *pneumophila*, while the remaining two comprised *L. pneumophila* subsp. *fraseri*. However, as the type strains of several serogroups (SGs), e.g., SG 8, SG 11 and SG 13, did not belong to any subgroup, it was presumed that more than six clonal populations might be found if isolates from other regions had been included. Therefore, the present study analysed a larger number of *L. pneumophila* isolates, collected from both Korea and Japan, in order to characterise the population structure of a more extensive set of genotypes.

MATERIALS AND METHODS

L. pneumophila isolates

In total, 268 epidemiologically unrelated isolates were studied, comprising 19 reference strains, 100 isolates from Japan, and 149 isolates from Korea. The Japanese isolates were collected between 1980 and 2002, and the Korean isolates were collected between 1985 and 2001. Twenty-one (18 Japanese and three Korean) were clinical isolates, while 228 were environmental isolates, comprising 159 (132 Korean and 27 Japanese) isolates from cooling tower water, 47 Japanese isolates from circulating hot water baths, nine Korean isolates from humidifiers, seven Japanese isolates from fountains, three Korean isolates from faucets, two Korean isolates from air-conditioners, and one Japanese isolate from a showerhead. Reference strains ATCC 33152, ATCC 33153, SF9, ATCC 43109, ATCC 33154, ATCC 33155, ATCC 33156, ATCC 33216, ATCC 33215, ATCC 33823, ATCC 35096, ATCC 35289, ATCC 43283, ATCC 443130, ATCC 43290, ATCC 43736, ATCC 43703, ATCC 35351 and GIFU13567 were used [15]. The Japanese isolates were provided by H. Miyamoto (University of Occupational and Environmental Health, Kitakyushu, Japan), and the Korean isolates by M.-Y. Park (Korean Center for Disease Control and Prevention, Seoul, Korea).

DNA extraction, PCR amplification and sequencing reactions were conducted as described previously [15]. Subspecies were identified according to the method described by Ko *et al.* [16], with some identifications being confirmed by 16S rRNA gene sequencing.

Phylogenetic analyses

The sequences of *rpoB* and *dotA* (300 bp and 360 bp, respectively) were determined as described previously [15]. Sequence analyses were performed with the MegAlign program v. 3.12e (DNASTAR, Madison, WI, USA). Phylogenetic trees were constructed by the neighbour-joining method, using the maximum-likelihood distance option of the HKY85 substitution model and no among-site rate variation. *Legionella micdadei* was employed as an outlier in the *rpoB* tree; a midpoint rooting option was used to root the *dotA* tree because of the lack of a reliable outlier. Subgroups (clusters) in the *rpoB* and *dotA* trees were defined as isolates that possessed *rpoB* and *dotA* sequence similarity values of >97% and >95%, respectively. Bootstrap values were evaluated with 1000 replications.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study were submitted to the GenBank database. The reference strains are designated by the following accession numbers: AY279999–AY280168 for *rpoB* and AY280169–AY280338 for *dotA*.

RESULTS

Subgrouping in the *rpoB* and *dotA* gene trees

Fig. 1 shows the gene trees of the Korean and Japanese isolates, based on the sequences determined. Because of space restrictions, only 19 reference strains and 68 isolates are shown; other isolates possessing identical sequences are not shown. Therefore, the overall topologies shown do not differ from the original gene trees, which included all of the isolates. In addition to the six subgroups (P-I to P-IV, F-I and F-II) described previously [15], four further subgroups (P-V to P-VIII) of *L. pneumophila* subsp. *pneumophila* were identified.

In the *rpoB* tree, subgroup P-V was related closely to subgroup P-I, while the other new subgroups (P-VI to P-VIII) were located at the basal position of the *L. pneumophila* subsp. *pneumophila* cluster (Fig. 1a).

The *dotA* tree topologies were similar to those described by Ko *et al.* [15]. The P-III subgroup was related closely to the *L. pneumophila* subsp. *fraseri* subgroups in the *dotA* tree (Fig. 1b). In addition, the P-VIII subgroup of *L. pneumophila* subsp. *pneumophila*, which was not detected among any of the Korean isolates, was also determined to be related to *L. pneumophila* subsp. *fraseri* (Fig. 1b). However, the P-V, P-VI and P-VII subgroups formed a large group with subgroups P-I and P-II. It has been suggested that such incongruent inter-relationships among subgroups constitute

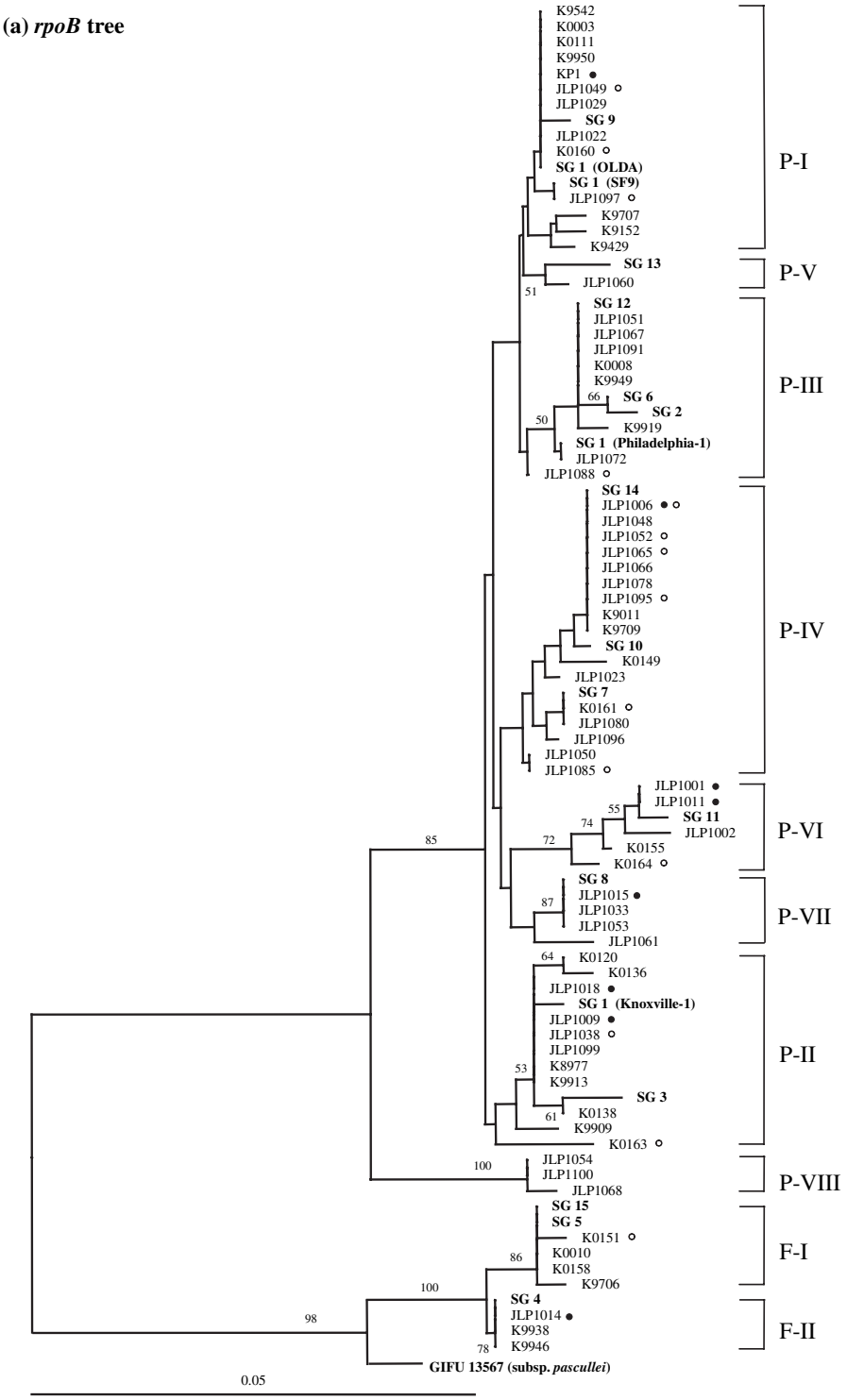

Fig. 1. Neighbour-joining trees inferred from (a) the *rpoB* and (b) the *dotA* sequences of Korean (K) and Japanese (JLP) *Legionella pneumophila* isolates. Reference strains are indicated by their serogroup numbers (SG 1–15). Subgroups (P-I to P-VIII and F-I and F-II) are indicated on the right; branch lengths are proportional to the number of nucleotide changes. Fourteen isolates that showed incongruent grouping between the *rpoB* and *dotA* gene trees are marked with open circles to the right of the isolate number; the clinical isolates are marked with closed circles. Only bootstrap values >50% in the bootstrap analysis are indicated at the corresponding branches.

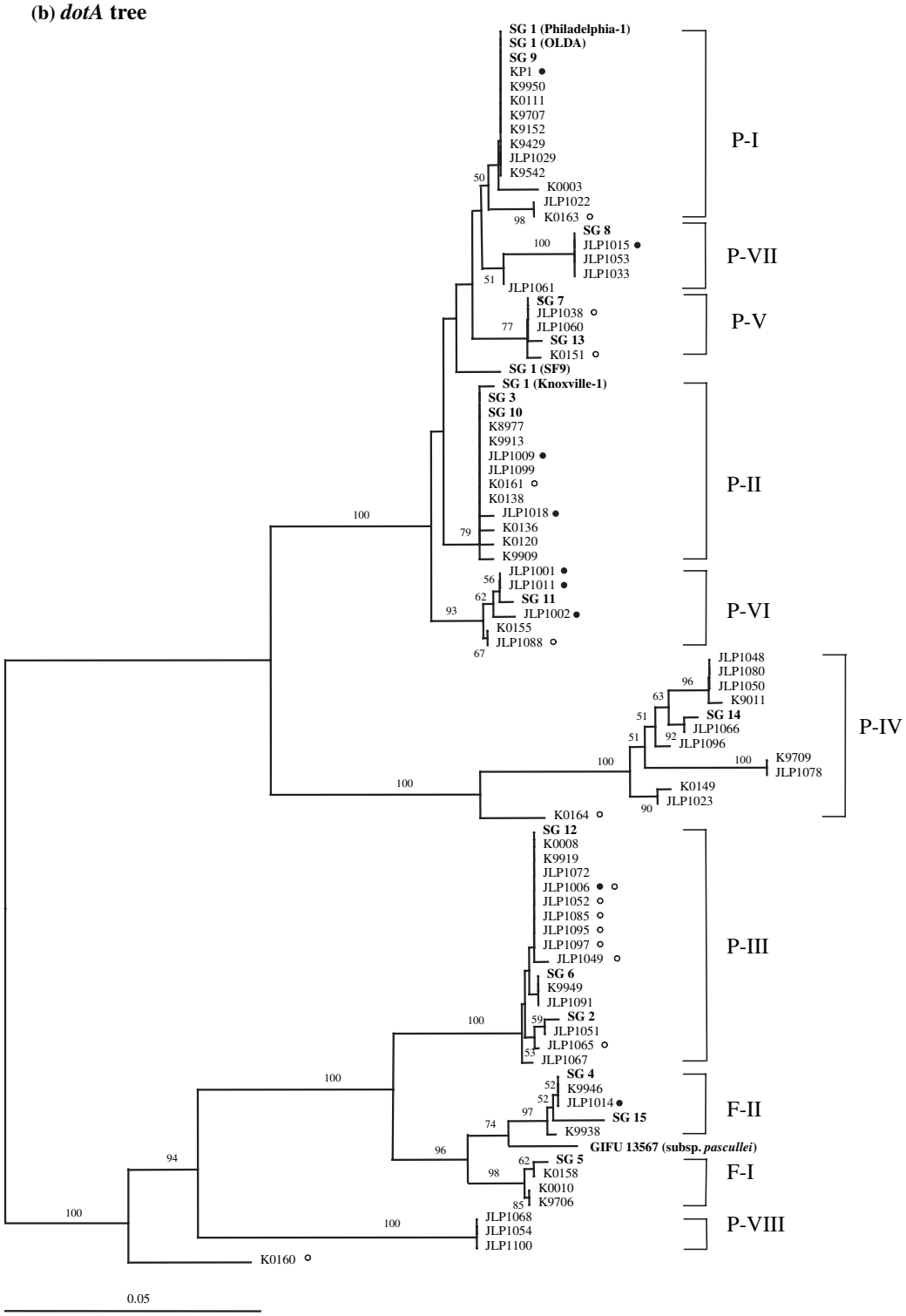

Fig. 1. (Continued)

evidence for horizontal gene transfer in *L. pneumophila* [15]. One isolate of *L. pneumophila* subsp. *pascullei* showed a close relationship with the F-II subgroup, and this was apparent in both trees.

Incongruent subgrouping of isolates in the *rpoB* and *dotA* trees

In previous studies [15,17], several reference strains have been shown to belong to different subgroups in different gene trees, but all of the Korean isolates showed consistent subgroupings. However, in the present study, 14 (5.6%) of 249 isolates belonged to different subgroups in the *rpoB* and *dotA* trees (Table 1). Incongruencies between the two gene trees were apparent for the five Japanese isolates (JLP1006, JLP1052, JLP1065, JLP1085 and JLP1095), which belonged to subgroup P-IV in the *rpoB* tree, but were in subgroup P-III in the *dotA* tree. One Korean isolate, K0151, was transferred from *L. pneumophila* subsp. *fraseri* (F-I) in the *rpoB* tree to *L. pneumophila* subsp. *pneumophila* (P-V) in the *dotA* tree, whereas isolate K0160, which belonged to subgroup P-I of *L. pneumophila* subsp. *pneumophila* in the *rpoB* tree, was relatively close to subgroups F-I, F-II, P-III and P-VIII in the *dotA* tree (Fig. 1b). JLP1022 and K0163, which in the *rpoB* tree belonged to subgroups P-I and P-II, yielded *dotA* sequences that were identical to those of subgroup P-VII (Fig. 1b). The sequences of the 14 isolates that belonged to different subgroups in the *rpoB* and *dotA* trees were confirmed by repeated sequencing.

Table 2 summarises the distribution of the *L. pneumophila* isolates in the *rpoB* and *dotA* gene trees (Table 2). Although 14 isolates exhibited incongruent topology in the gene trees, no significant differences ($p > 0.05$) were found between the subgroup distributions based on the *rpoB* and *dotA* sequences. Most isolates, 39.4% and 38.3% in the *rpoB* and *dotA* trees, respectively, belonged to the P-I subgroup, followed by subgroups P-II, P-III and P-VI.

Table 1. *Legionella pneumophila* isolates that belonged to different subgroups in the *rpoB* and *dotA* trees

Strain	Origin	Source	*rpoB*	*dotA*
JLP1006	Hyogo, Japan	Clinical isolate	P-IV	P-III
JLP1038	Fukuoka, Japan	Cooling tower water	P-II	P-V
JLP1049	Fukuoka, Japan	Circulating public bath hot water	P-I	P-III
JLP1052	Fukuoka, Japan	Circulating public bath hot water	P-IV	P-III
JLP1065	Fukuoka, Japan	Circulating public bath hot water	P-IV	P-III
JLP1085	Fukuoka, Japan	Circulating public bath hot water	P-IV	P-III
JLP1088	Fukuoka, Japan	Circulating public bath hot water	P-III	P-VI
JLP1095	Fukuoka, Japan	Circulating public bath hot water	P-IV	P-III
JLP1097	Fukuoka, Japan	Circulating public bath hot water	P-I	P-III
K0151	Ulsan, Korea	Cooling tower water	F-I	P-V
K0160	Busan, Korea	Cooling tower water	P-I	ND
K0161	Busan, Korea	Cooling tower water	P-IV	P-II
K0163	Busan, Korea	Cooling tower water	P-II	P-I
K0164	Busan, Korea	Cooling tower water	P-VI	P-IV

ND, not determined (i.e., did not belong to any subgroup).

Subgroup distributions of the Korean and Japanese isolates

Most (>53%) of the Korean isolates belonged to subgroup P-I, compared with only 18.0% (by *rpoB* sequencing) and 15.0% (*dotA*) of the Japanese isolates. However, because the isolates were collected from such different sources, it was impossible to make direct comparisons between the *L. pneumophila* subgroup distributions in the two countries. Therefore, only isolates obtained from cooling tower water, a common source of the *L. pneumophila* isolates used in this study, were compared (Table 2). Most (> 70%) of the Korean isolates belonged to subgroups P-I and P-II. Many of the Japanese isolates from cooling tower water also belonged to subgroup P-I, but

Table 2. Subgroup distribution of Korean and Japanese *Legionella pneumophila* isolates from cooling tower water

	subsp. *pneumophila*, *n* (%)								subsp. *fraseri*, *n* (%)	
Gene/origin/source	P-I	P-II	P-III	P-IV	P-V	P-VI	P-VII	P-VIII	F-I	F-II
rpoB/Total ($n = 249$)[a]	98 (39.2)	43 (17.3)	31 (12.4)	30 (12.0)	4 (1.6)	8 (3.2)	8 (3.2)	7 (2.8)	13 (5.2)	7 (2.8)
Cooling tower water ($n = 159$)[b]										
Korean ($n = 132$)	71 (53.8)	22 (16.7)	12 (9.1)	6 (4.5)		4 (3.0)			13 (9.8)	4 (3.0)
Japanese ($n = 27$)	13 (48.1)	1 (3.7)	6 (22.2)	4 (14.8)			3 (11.1)			
dotA/Total ($n = 248$)[a,c]	95 (38.2)	41 (16.5)	37 (14.9)	25 (10.1)	6 (2.4)	8 (3.2)	10 (4.0)	7 (2.8)	13 (5.2)	6 (2.4)
Cooling tower water ($n = 158$)[b,c]										
Korean ($n = 131$)[c]	71 (54.2)	21 (16.0)	12 (9.2)	6 (4.6)	1 (0.8)	3 (2.3)	1 (0.8)		13 (9.9)	3 (2.3)
Japanese ($n = 27$)	12 (44.4)		6 (22.2)	4 (14.8)	1 (3.7)		4 (14.8)			

[a]No significant difference ($p > 0.05$) in subgroup distributions between *rpoB* and *dotA* analysis.
[b]No significant difference ($p > 0.05$) in subgroup distributions between the two countries.
[c]K0160 was excluded in this analysis because it could not be included in any subgroup and might belong to a new subtype in the *dotA* gene tree.

fewer belonged to subgroup P-II. None of the Japanese cooling tower water isolates belonged to the F-I or F-II subgroups of *L. pneumophila* subsp. *fraseri*, while 13 (9.8%) of the Korean isolates belonged to subgroup F-I in both the *rpoB* and *dotA* trees, and four (3.0%) and three (2.3%) isolates belonged to subgroup F-II in the *rpoB* and *dotA* trees, respectively. However, the small number of Japanese cooling tower water isolates meant that the difference in the subgroup distributions was not statistically significant ($p > 0.05$).

Because of the ubiquitous nature and regional dependence intrinsic to particular clonal groups, the Japanese *L. pneumophila* isolates were analysed on the basis of isolation sources. Isolates from four different sources showed consistent highly significant differences ($p < 0.01$) with regard to subgroup distribution on the basis of both *rpoB* and *dotA* analyses (Table 3). Isolates from circulating hot water in public baths were identified primarily as belonging to subgroups P-III and P-IV, whereas almost half of the isolates from cooling tower water belonged to the P-I subgroup. The distribution of the isolates from fountains was similar to the distribution of the cooling tower water isolates. In addition, seven isolates of subgroup P-VIII, which contained none of the reference strains, were all collected from circulating hot water at a public bath.

The present study included 21 clinical isolates. Of the 18 Japanese isolates, 15 belonged to *L. pneumophila* subsp. *pneumophila*, and three belonged to the F-II subgroup of *L. pneumophila* subsp. *fraseri* (Table 3). The three Korean clinical isolates belonged to subgroup P-I [15], whereas only one (5.6%) of the 18 Japanese clinical isolates belonged to this subgroup. In addition, the 17 Korean isolates of *L. pneumophila* subsp. *fraseri* were all environmental isolates, whereas the three Japanese isolates belonging to *L. pneumophila* subsp. *fraseri* were all clinical isolates.

DISCUSSION

The *Legionella* genus includes a group of ubiquitous microorganisms, among which lateral gene transfer occurs commonly [18]. In natural environments, this can result in the emergence of specific clones in particular areas or under certain environmental conditions. It has also been suggested that particular types of *L. pneumophila* in specific regions might be related to disease [18], and reports on other pathogens have drawn such correlations between particular clonal groups and human disease [19,20]. By understanding bacterial population structures and dynamics at the genetic level, it may be possible to obtain deeper insights into the origins and transmission of bacterial disease [21], and thus establish more effective public health policies [22]. Subgrouping based on population genetic studies may provide more precise information on the identities of *L. pneumophila* isolates compared with serological grouping.

Multilocus sequence typing analyses of *L. pneumophila* populations, using two virulence genes (*dotA* and *mip*) [23], two surface protein genes (*mip* and *mompS*) and three housekeeping genes (*can*, *asd* and *rpoB*) [24] have been reported. In the present study, *dotA* and *rpoB* sequences were used to divide *L. pneumophila* isolates into several subgroups, which were then compared on the basis of the sources of isolation. Although only two marker genes were employed, these findings suggest that subgrouping based on *rpoB* and *dotA* sequences is useful

Table 3. Subgroup distribution of *Legionella pneumophila* isolates from Japan

		subsp. *pneumophila*, *n* (%)								subsp. *fraseri*, *n* (%)	
Gene	**Source**[a]	**P-I**	**P-II**	**P-III**	**P-IV**	**P-V**	**P-VI**	**P-VII**	**P-VIII**	**F-I**	**F-II**
rpoB	Circ. pub. bath[b] (*n* = 48)	2 (4.2)	5 (10.4)	12 (25.0)	19 (39.6)	1 (2.1)		2 (4.2)	7 (14.6)		
	Cooling tower (*n* = 27)	13 (48.1)	1 (3.7)	6 (22.2)	4 (14.8)			3 (11.1)			
	Fountain (*n* = 7)	2 (28.6)	1 (14.3)	1 (14.3)		1 (14.3)		2 (28.6)			
	Clinical isolates (*n* = 18)	1 (5.6)	5 (27.8)		1 (5.6)	2 (11.1)	4 (22.2)	2 (11.1)			3 (16.7)
dotA	Circ. pub. bath[b] (*n* = 48)		5 (10.4)	17 (35.4)	15 (31.3)	1 (2.1)	1 (2.1)	2 (4.2)	7 (14.6)		
	Cooling tower (*n* = 27)	12 (44.4)		6 (22.2)	4 (14.8)	1 (3.7)		4 (14.8)			
	Fountain (*n* = 7)	2 (28.6)	1 (14.3)	1 (14.3)		1 (14.3)		2 (28.6)			
	Clinical isolates (*n* = 18)	1 (5.6)	5 (27.8)	1 (5.6)		2 (11.1)	4 (22.2)	2 (11.1)			3 (16.7)

[a]Significant difference ($p < 0.01$) in subgroup distributions depending on isolate sources.
[b]Circulating public bath hot water. One isolate (JLP1031) from a showerhead was included in this category.

in *L. pneumophila* population studies. Although the description of population structure on the basis of a single virulence gene (*dotA*), which is not neutral to selection, might appear to be somewhat questionable, the results obtained were generally consistent with subgrouping based on *rpoB*.

As shown by the subgroups found in the present study, *L. pneumophila* consists of clonal subspecies, which could also be identified by multi-enzyme electrophoresis [25]. The inclusion of Japanese isolates allowed four new subgroups, designated P-V to P-VIII, to be identified. In the *rpoB* tree, subgroups P-V, P-VII and P-VIII were found exclusively in the Japanese isolates, while P-VI was found in both the Korean and Japanese isolates. As the sources of the isolates differed between the two countries, it might have been expected that new subgroup populations would be identified among the Japanese isolates. The predominant clonality was observed to depend on the source of the isolates. Furthermore, although not statistically significant, the subgroup distributions of the isolates from cooling tower water were slightly different between the two countries. More data concerning Japanese cooling tower water isolates will be required to confirm the findings of the present study.

Subgroup clonality appeared to be maintained among the Japanese isolates from different environmental sources. For example, the P-I subgroup was the principal subgroup among the isolates from the Japanese cooling tower water, whereas P-III and P-IV predominated among the isolates from circulating hot water at a public bath. In addition, the F-I subgroup of *L. pneumophila* subsp. *fraseri* was not detected among the Japanese isolates. These results suggest that *L. pneumophila* may develop a unique genetic population structure within a particular region or environment.

To date, there is no available evidence to suggest that any particular *L. pneumophila* type or clone has greater pathogenicity than another. Although it was not possible to correlate the ten subgroups defined in the present study with the pathogenicity of *L. pneumophila*, it was notable that 50% of the Japanese clinical isolates were distributed between subgroups P-II and P-VI (Table 3), and were related closely in the *dotA* tree (Fig. 1b), and that all three of the Japanese isolates of *L. pneumophila* subsp. *fraseri* in subgroup F-II were clinical isolates, rather than environmental isolates. In addition, although they had been isolated from different provinces in Japan (Kyushu and Kanto) in different years (1980 and 1990), four of the clinical isolates (JLP1001, JLP1002, JLP1011 and JLP1020) were of subgroup P-VI, which included only one hot-bath water isolate (JLP1088) among the Japanese environmental isolates in the *dotA* tree. Although Alli *et al.* [26] demonstrated that the virulence or pathogenic traits of *L. pneumophila* are heterogeneous, further study will be required in order to ascertain whether differences in the pathogenicity of *L. pneumophila* are related to these subgroupings.

Further evidence of horizontal gene transfer within the *L. pneumophila* population was obtained during the present study. The position of subgroup P-III in the *dotA* tree is suggestive of horizontal transfer of *dotA* from *L. pneumophila* subsp. *fraseri* to *L. pneumophila* subsp. *pneumophila* (Fig. 1b). In previous studies [15,17], all 79 of the Korean isolates had identical subgroupings in both gene trees, although several reference strains had incongruent subgroupings. However, in the present study, the subgroupings of 14 of the isolates differed in the *rpoB* and *dotA* trees (Table 1). Surprisingly, evidence of gene exchange between two subspecies (*L. pneumophila* subsp. *pneumophila* and *L. pneumophila* subsp. *fraseri*) that are generally considered to represent genetically separate entities was observed for isolate K0151 (Table 1). It has been reported previously that the *dot/icm* locus can be transferred horizontally via chromosomal conjugation [27]. Several isolates belonging to the P-IV subgroup, which is the most diverse subgroup within the *L. pneumophila* population, also showed clear incongruent interrelationships (data not shown). Moreover, the incongruent subgroupings of recent isolates support the notion that intraspecific recombination occurs in nature [17].

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