Genetic diversity of poultry red mites (*Dermanyssus gallinae*) in Japan and prevalence of their avian pathogens

（日本国内におけるワクモ（***Dermanyssus gallinae***）の
遺伝的多様性と病原体保有状況）

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ABBREVIATIONS

16S rRNA: 16S ribosomal ribonucleic acid

APV: avian pox virus

COI: mitochondrial cytochrome *c* oxidase subunit I

DNA: deoxyribonucleic acid

ER: *Erysipelothrix rhusiopathiae*

FAdV: fowl adenovirus

ITS: nuclear internal transcribed spacer

LTR: long terminal repeat

MDV: Marek's disease virus

MS: *Mycoplasma synoviae*

MG: *Mycoplasma gallisepticum*

NJ: Neighbor - Joining

ORF: open reading frame

PCR: polymerase chain reaction

PRM: poultry red mite

RE provirus: reticuloendotheliosis provirus

SE: *Salmonella enterica*

TR2: terminal repeat 2

GENERAL INTRODUCTION

Taxonomy

Dermanyssus gallinae was first described by De Geer in 1778. It is a blood-feeding arthropod parasite belongs to the subclass Acari, superorder parasitiformes, order Mesostigmata (Gamasida), in the family Dermanyssidae [15]. It becomes red if it has recently fed on its host's blood, so it is called poultry red mite (PRM) as a common name.

Life cycle

D. gallinae progresses through five life stages: egg, larva, protonymph, deutonymph and adult [31]. PRM eggs are deposited in cracks or crevices in the poultry house and hatch into larvae in 2 to 3 days. The larvae molt into protonymphs in 1 to 2 days without feeding. The larva has three pairs of legs, but from the protonymph stage, it has four pairs of legs [15]. The protonymphs have blood meal and then molt into deutonymphs in another 1 to 2 days. A second blood meal is required for the deutonymphs to become adults in 2 to 3 days. The whole development takes about a week under optimal conditions [53].

Behavior and Ecology

D. gallinae feed every 2 to 4 days and usually spend up to an hour on the host. The mites are rarely seen on the birds because they typically feed at night [53]. Adult

mites spend most of the daytime away from the host and can survive several months without feeding. During the day, mites can be found in cracks and litter in the poultry house. *D. gallinae* can grow at temperatures between 10 and 37ºC. Fecundity is greatest at 30ºC [31].

Morphology

Adult *D. gallinae* is approximately 1.5 mm in length [53]. All other developmental stages are smaller. The adult mite possesses a prominent dorsal shield. The shield does not quite extend to the posterior end of the body and its posterior end is truncated. The hairs on the dorsal shield are smaller than those on the carapace that surrounds the shield. The mite's posterior ventral surface has a prominent anal plate. The anus is located on the posterior aspect of this plate. The mites possess long, whip-like chelicerae which are slender with small scissor-like parts at the ends [11].

Host range

D. gallinae is a blood-sucking parasite primarily on domestic poultry as well as in wild birds [15]. The mites normally feed around the breast and legs in poultry, especially in laying hens. In the absence of an avian host, it occasionally attacks mammals, such as dogs, cats, rodents, horses and even human [1, 40, 47, 51].

Clinical signs

Infestation with *D. gallinae* causes feather loss, pain, irritation, decrease in egg

production, and increased pustules scabs and hyperpigmentation. Subsequent serious infestation with a large number of PRM causes anemia and sometime death [26]. Moreover, insufficient immune-responses against several PRM vaccines and immunosuppressive effects associate with their infestations are pointed out [21, 22]. Actually, down regulation of Th1 and pro-inflammatory cytokines/chemokines were reported in chickens infested with the mite [1]. In addition, human reactions to PRM included dermatitis, irritation, pruritus and other skin lesions [2].

D. gallinae is also a potential mechanical vector of several pathogens in poultry [58], such as *Salmonella enterica* (SE) [20, 57], *Erysipelothrix rhusiopathiae* (ER) [8], *Pasteurella multocida* [36] and avian pox virus (APV) [7, 48]. Particularly, *D. gallinae* has been shown to take up and transmit *Salmonella* entertidis between chicken under experimental conditions, suggesting it is also capable of spreading salmonellosis under field conditions [57].

Prevention

Prevention of *D. gallinae* populations in poultry farms is key to ensuring the welfare of poultry, although eradication of *D. gallinae* from a poultry house is extremely difficult [53]. Control measures for PRM have typically relied on acaricides [9, 16]. However, repeated long-term use of the compounds has resulted in the development of drug-resistant populations of PRM. Vaccine development for PRM has been attempted [3, 21, 23] as another way for preventing the mite. However, efficient vaccine has not been developed yet. To date, beside using acaricides, improving hygiene practices such as regular poultry house cleaning are generally underestimated as PRM control measures.

Distribution

D. gallinae is worldwide in its distribution [41]. Especially, *D. gallinae* is one of the serious problems as its massive infestation in poultry farms in the North and South America [15], in Europe such as France, Denmark, Norway, Scotland, Netherlands, Finland, Belgium [35], Sweden [24], Poland [60] , UK [12, 19], and Italy [28], and Africa [17, 33]. In Asia, the PRM was reported as one of the common ectoparasites in poultry farms in Iran [38] and China [59]. In Japan, PRM is also considered as the most problematic ectoparasite in layer farms [34, 52].

Genetic diversity

Recently, DNA sequences of various genomic regions such as the mitochondrial cytochrome *c* oxidase subunit I (COI), nuclear internal transcribed space (ITS) and 16S rRNA of *D. gallinae* distributed in different regions or countries were examined. The nucleotide sequence analysis revealed genetic diversity of the COI gene at least 4 groups, A, B, C and D. The ITS sequence analysis revealed differences between PRM in domestic and wild birds [4]. Although PRM is distributed almost worldwide, molecular studies have so far examined only mite populations in European countries. Almost no genetic information of the mite is available in other parts of the world.

Aim of the Dissertation

D. gallinae is distributed worldwide and is reported as a potential vector for some infectious diseases [58]. This implies that some pathogens may be spread with the mite in European countries. However, similar phylogenetic studies, which are essential for understanding the epidemiology of the mite, have not been conducted in Asian countries. In addition, a comprehensive study of the distribution of avian pathogens in PRM has not been conducted.

In the present study, the phylogenetic and genetic diversity of mites distributed in Japan, and genetic relationships with the mites in other countries, were examined. Furthermore, an attempt was made to detect several avian pathogens in the mite. These studies will provide epidemiological information that will help to understand the role of PRM in distribution of avian pathogens.

CHAPTER 1

Molecular epidemiological characterization of poultry red mite, *Dermanyssus gallinae*, in Japan

Introduction

Dermanyssus gallinae (De Geer, 1778), order Mesostigmata, Acari, also known as the PRM is an obligatory blood-sucking parasite of both domestic and wild birds [15, 42]. In poultry farms, it causes irritation, loss of weight, reduction in egg production, anemia and death, leading to economic loss in the poultry industry [6].

D. gallinae is distributed throughout the world [41], and their genetic diversity based on nucleotide sequences has been examined in Europe, the United States, Brazil and Australia. The genes sequenced include mitochondrial cytochrome *c* oxidase subunit I (COI) gene [29, 35, 44], 16S rRNA [43, 44], and the nuclear internal transcribed spacers (ITS) region [4, 28, 35, 37, 44]. These molecular epidemiological studies revealed intra- and international migration of the mite in these countries. However such phylogenetic studies have not yet been conducted in Asian countries including Japan. In this study, the COI, 16S rRNA and ITS sequences of mite samples collected throughout Japan were analyzed. These sequences were compared with sequences obtained in other countries to reveal the genetic diversity of *D. gallinae*.

Materials and Methods

Dermanyssus gallinae

A total of 239 samples of *D. gallinae* were collected from 226 chicken farms from 2005 to 2012 in 40 prefectures in Japan. All samples were fixed in 99.5% ethanol and stored at room temperature until use.

DNA extraction

DNA was prepared from the whole body of each individual adult mite obtained from each sample. The mite was homogenized with zirconia beads using TissueLyser II (Qiagen Inc., Chatsworth, CA, U.S.A.) in 20 *μl* of buffer 1 provided by Ten Minute DNA Release Kit-1 (Jacksun Easy Biotech Inc., NY, U.S.A.) and DNA samples were prepared according to the manufacturer's instructions. The DNAs were stored at -30 ºC until use.

Polymerase chain reaction (PCR)

A part of nucleotide sequence of mitochondrial COI gene was amplified by using the primers forward CO1Fyuw114 (5'-AGATCTTTAATTGAAGGGGG -3') and reverse CO1Ryuw114 (5'-AAGATCAAAGAATCGGTGG-3') designed in this study. The amplified fragment corresponded to nucleotide position from 61 to 742 of COI gene, accession number AM921853. A part of nucleotide sequence of 16S rRNA gene was amplified by using pair of primers forward Rh16S (5'-

GCTCAATGATTTTTTAAATTGCTG-3’) and reverse Rh16S (5’-CCGGTCTGAACTCAGATCATG-3’) [10]. The expected length of PCR product was 444-bp, corresponds to nucleotide position from 3 to 446 of 16S rRNA gene, accession number L34326. A part of nucleotide sequence of ITS region including ITS1, 5.8S rRNA and ITS2 sequences was amplified by using the primers forward ITS-Fyuw125 (5’-AGTCGTAACAAGGTTTCCG-3’) and reverse ITS-Ryuw125 (5'-TCCTCCGCTTATTGATATGC-3’) designed in this study. The expected length of PCR product was 561-bp, corresponds to nucleotide position from 97 to 657 of ITS region, accession number GQ129212.

PCR was performed in a volume of 25 μl containing 12.5 μl of Hot start GoTaq (Promega, WI, U.S.A.), 1.0 μl mixture of forward and reverse primers (final concentration was 0.5 μM each) and 2 μl of DNA template (for amplification of COI and ITS) or 1 μl (for amplification of 16S rRNA gene). The PCR for COI gene was conducted in one cycle of 95ºC for 2 min, 40 cycles of 95ºC for 20 sec, 55ºC for 30 sec, 72ºC for 1 min and a final cycle of 72ºC for 4 min. The PCR for 16S rRNA was performed in accordance with the previous study [10]. PCR for ITS was conducted in one denaturation step at 95ºC for 4 min and followed by 40 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72ºC for 45 sec. The final cycle was 72ºC in 7 min. After the amplification, reaction mixtures were subjected to electrophoresis in 1.2% (w/v) agarose gels and stained with 0.1% GelRed (Biotium, Inc., Hayward, CA, U.S.A.). The obtained PCR amplicons were excised and purified by using MinElute Gel Extraction Kit (Qiagen, Germany) for the nucleotide sequence analysis.

Nucleotide sequencing

Nucleotide sequences were determined using Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, U.S.A.) according to the manufacturer's instructions. Nucleotide sequences were assembled and manually edited by using CodonCode Aligner 3.6.1 (CodonCode, Dedham, MA, U.S.A.). After elimination of primer sequences from the obtained sequences, the resultant sequences were analyzed as described below.

Sequence analysis

The determined sequences of COI and 16S rRNA genes were assigned to their haplotypes. Mites with identical sequences were designated as one haplotype.

For the phylogenetic analysis of COI sequences obtained in this study, 476-bp covered from the position from 121 to 596 of the sequence accession number AM921853 were used because this sequence region is most commonly reported in the database. In this study, the 926 COI sequences, detected in European countries, U.S.A., Brazil and Australia, obtained from the database were also used. In the case of 16S rRNA sequence, 299-bp corresponding to the sequence of *D. gallinae* (accession number L34326) at the position from 103 to 401 was used for the phylogenetic analysis to reveal the phylogenetic relationship in the world, because this is the region most frequently reported. In this study, seventeen 16S rRNA sequences detected in European countries, obtained from the database were used.

The nucleotide sequences were aligned using ClustalX with default settings [2]. Subsequently, the phylogenetic trees were constructed by the Neighbor-Joining (NJ) method [45] with the Kimura two-parameter option. A bootstrap analysis was done with 1,000 replicates.

Results

Nucleotide sequence analysis of COI gene

The nucleotide sequences of 643-bp of COI gene of 101 mite samples obtained in Japan (accession numbers LC029457-LC029557) were classified into 28 haplotypes (Table 1).

In a phylogenetic tree based on the 476-bp COI nucleotide sequence (Fig. 1), mites from Japan and other countries clustered into 4 haplogroups A, B, C and D as previously reported [35]. All 28 haplotypes found in Japan clustered in either haplogroup A or B. These haplotypes diverged into two sub-lineages designated as sub-haplogroups AJ1 and AJ2, and BJ1 and BJ2, respectively. AJ1 consisted of 14 haplotypes (AJ1.1-AJ1.14), AJ2 consisted of two haplotypes (AJ2.1 and AJ2.2), BJ1 consisted of six haplotypes (BJ1.1-BJ1.6) and BJ2 consisted of six haplotypes (BJ2.1-BJ2.6). Sample names and sample numbers classified into each haplotype are shown in Table 1.

The sequences of 14 and 6 haplotypes in AJ1 and BJ2, respectively, were found only in Japan. Phylogenetic analysis showed that the sequences of haplotypes in AJ1 and BJ2 were relatively distant from the sequences detected in other countries. On the other hand, the sequences of haplotypes in AJ2 and BJ1 were closely related to the sequences previously detected in several European countries (Fig. 1). The 476-bp sequence of haplotype AJ2.1 was identical to the sequence of A12 haplotype detected in Norway [35]. In addition, the 6 haplotypes in BJ1 were closely related to 11 reported haplotypes, B3 to B13 of haplogroup B [35], and the 476-bp sequences of haplotypes BJ1.2, BJ1.3 and BJ1.4 were identical to the sequence of haplotype B7

distributed in Sweden, Netherlands and Poland, and B8 distributed in Scotland [35].

Nucleotide sequence analysis of 16S rRNA

The 397-bp 16S rRNA sequences of the 239 samples (accession numbers LC029560-LC029798) were determined and were classified into 26 haplotypes (Table 1). In a phylogenetic tree constructed with the 299-bp of the 397-bp sequence, which was most commonly deposited in the database, these haplotypes diverged into two lineages, each having two clades (Fig. 2).

The phylogenetic relationships of 101 mite samples, which were used for both 16S rRNA and COI analyses, in the 16S rRNA tree were consistent with the relationships among haplogroups and sub-haplogroups found in the phylogenetic tree constructed with the COI sequences. Because the same phylogenetic relationships were found in both trees, the two lineages and four clades observed in the 16S rRNA phylogenetic tree were designated as haplogroups A and B, and sub-haplogroups AJ1 and AJ2, and BJ1 and BJ2, respectively (Fig. 2), according to the COI tree.

AJ1 in this tree consisted of 12 haplotypes (AJ1.1 to AJ1.12) and AJ2 consisted of 5 haplotypes (AJ2.1 to AJ2.5). BJ1 consisted of 3 haplotypes (BJ1.1 to BJ1.3) and BJ2 consisted of 6 haplotypes (BJ2.1 to BJ2.6). Of the 239 sequences examined, 130 sequences belonged to the haplotype AJ1.1 of 16S rRNA. This haplotype was the most frequently found in Japan. In the B haplogroup, BJ2.1with 39 sequences and BJ1.1 with 26 sequences were the most common haplotypes.

The haplotypes in AJ2 and BJ1 clustered with the sequences detected in European countries, respectively (Fig. 2). The 299-bp sequences of haplotypes AJ2.1, AJ2.4 and AJ2.5 were identical to the sequences detected in France (accession

number AM921890), Norway (accession number AM921884), Denmark (accession number AM921887) and Netherlands (accession number FM207492). The sequences of haplotypes BJ1.1 and BJ1.2 were identical to the sequences detected in Norway (accession number AM921883) and Poland (accession number AM921914). On the other hand, all haplotypes in sub-haplogroup BJ2 were relatively distant from the sequences reported in European countries. In the case of the haplotypes in sub-haplogroup AJ1, one sequence obtained from Spain (accession number AM921885) clustered with AJ1 sub-haplogroup. However, no sequences found in Japan were identical to this sequence.

Distribution of the sub-haplogroups of the mite in Japan

Geographical distribution of sub-haplogroups determined based on COI and 16S rRNA sequences were examined (Fig. 3). Based on the 16S rRNA sequences, sub-haplogroup AJ1 was distributed all over Japan (30/40 studied prefectures), while mites in AJ2 were found only in two marginal prefectures, Hyogo and Okayama. Mites in BJ1 were found in 14 prefectures located through Japan. Although the number is relatively restricted, these prefectures were located all over Japan, from the most northerly prefecture (Hokkaido) to the most southerly prefectures (Saga). Mites in BJ2 were detected over an area on the west side from Chiba prefecture in Japan (21/40 studied prefectures).

Nucleotide sequence analysis of the ITS region

Alignment of the 522-bp of ITS region of 33 samples (accession numbers

LC034921-LC034953) revealed that there are two sequence types that differ in two positions corresponding to 199 and 516 of the reported ITS sequence (accession number GQ129212). Positions 199 and 516 are located in the ITS1 and ITS2 regions, respectively. Of the 33 samples, 25 samples of both positions at 199 and 516 were C (type I) and the other 8 samples were T and A (type II), respectively (Table 1). The type II sequences obtained from the 8 samples were identical to the sequence of ITS region of the mites collected in Sweden (accession number GQ12912), Denmark (accession number AM903303) and Norway (accession number AM931072). On the other hand, none of the sequences were identical to the type I sequence. However, two sequences, accession numbers AM903308 (from Italy) and AM930889 (from France), were identical except for one N (indicating any nucleotide) in each sequence at a different position.

All mite samples which showed the same ITS sequence were classified into the same haplotype except for four samples (Table 1). The ITS sequences of two samples collected in Hokkaido (type I) and Iwate (type II) prefectures are different but their haplotypes of COI (BJ1.2) and 16S rRNA (BJ1.1) are the same. Similarly, the ITS sequences of two other samples collected in Chiba prefecture are different but their 16S rRNA haplotype is BJ2.1 (COI haplotype was not determined).

Discussion

In this study, *D. gallinae* were harvested from 40 prefectures all over Japan, and parts of their COI gene and 16S rRNA sequences were determined. The sequence analyses revealed that the haplotypes in AJ2 and BJ1 sub-haplogroups in both the COI and 16S rRNA trees were closely related to the sequences of haplotypes found in Europe. Although it is unclear which countries were the origins of these haplotypes, the findings indicate that the mites of these haplotypes distributed in Japan are genetically related to the mites in European countries. In the case of the haplotypes in the AJ2 sub-haplogroup, the distribution in Japan was restricted to two adjacent prefectures. Because the mites can be easily spread with the transportation of infested chickens or related materials among poultry farms, such a restricted distribution implies that the haplotypes in AJ2 were recently introduced to Japan.

The sequences of the haplotypes in the AJ1 sub-haplogroup were different from all of the other sequences and phylogenetically distant from the mites detected in other countries. In addition, the haplotypes in the AJ1 sub-haplogroup were found most frequently and were most widely distributed in Japan. These findings imply that mites with haplotypes in the AJ1 sub-haplogroup are indigenous, and may have independently evolved in Japan. In the case of the haplotypes in the BJ2 sub-haplogroup, their sequences were also different from sequences reported in other countries. However, their distribution, in contrast to the distribution of AJ1, was restricted to western Japan. Because the mites can be easily spread as mentioned above, it seems more likely that the haplotypes in BJ2 are not indigenous but were relatively recently introduced to western Japan from other countries where COI or 16S rRNA sequences of mites have not yet been determined. Of the haplotypes in

the BJ2 sub-haplogroup, BJ2.1 haplotypes of COI and 16S rRNA were most frequently detected. This finding implies that the haplotypes of the BJ2 sub-haplogroup found in this study might have diverged from the BJ2.1 haplotype in Japan.

Most of the mite samples that had the same ITS sequences were classified into the same haplotypes. However, the ITS sequences of some samples were different from each other but their haplotypes were the same. Because both COI and 16S rRNA are mitochondrial genes and the ITS region is a nuclear gene, these sequences are useful for revealing hybridization histories between different lineages [41]. The incongruence between the haplotype based on the mitochondrial gene sequence and the sequence of nuclear genes found in this study suggests that a hybridization event occurred between different haplotypes of the mites.

This study revealed genetic diversities of *D. gallinae* distributed in Japan. In addition, phylogenetic relationships among the mites in Japan and other countries indicate the possibility of overseas transmission of the mites. However, it should be noted that nucleotide sequences of *D. gallinae* have been determined for only a few areas despite their worldwide distribution. For a comprehensive understanding of intra- and international migrations of *D. gallinae*, further sequence analyses of the mites collected from all over the world are needed.

Table 1. *D. gallinae* samples used in this study, and their haplogroups, sub-haplogroups and haplotypes based on the nucleotide sequences of 16S rRNA , COI and ITS region

COI			16S rRNA Haplotype [c)]	Sample name (Prefecture/ID/Year)	Sample number
Haplogroup [a)]	Sub-haplogroup	Haplotype [b)]			
A	AJ1	AJ1.1	AJ1.1	Aichi/27/2004, Chiba/63/2006, Chiba/132/2009, **Chiba/176/2010**, Chiba/190/2010, **Chiba/197/2010**, Fukui/195/2010, Gunma/93/2007, Gunma/95/2007, Gunma/121/2008, Ibaraki/144/2009, Iwate/149/2009, Iwate/182/2010, **Mie/199/2010**, Miyazaki/172/2010, **Miyazaki/173/2010**, Saitama/82/2007, Saitama/88/2007, Tochigi/105/2008, Tochigi/109/2008, **Tottori/185/2010**, Toyama/196/2010, Yamagata/193/2010, Yamaguchi/102/2008	24
			AJ1.10	**Chiba/165/2010**	1
			AJ1.11	Chiba/143/2009, Chiba/221/2011	2
			AJ1.12	Gunma/184/2010	1
			AJ1.6	Niigata/111/2008, Niigata/113/2008	2
		AJ1.2	AJ1.1	**Miyazaki/171/2010**	1
		AJ1.3	AJ1.1	**Gunma/183/2010**	1
		AJ1.4	AJ1.1	Saitama/75/2007	1
		AJ1.5	AJ1.1	Kanagawa/156/2009	1
		AJ1.6	AJ1.1	Chiba/202/2010, Kagoshima/163/2010	2
		AJ1.7	AJ1.3	***Nagasaki/H2/2010***	1
		AJ1.8	AJ1.1	**Chiba/191/2010**	1
		AJ1.9	AJ1.1	Chiba/73/2007, Chiba/114/2008, Chiba/130/2009, Chiba/133/2009, Chiba/158/2010, Chiba/164/2010, Chiba/204/2011, Gunma/90/2007, Gunma/96/2007, Kanagawa/155/2009, **Miyagi/198/2010**, Tochigi/147/2009, Chiba/60/2006	13
			AJ1.5	Gunma/91/2007	1
		AJ1.10	AJ1.1	Chiba/67/2006	1
		AJ1.11	AJ1.1	**Gunma/181/2010**, Ibaraki/47/2005	2
		AJ1.12	AJ1.1	Chiba/159/2010	1
		AJ1.13	AJ1.1	Niigata/15/2004	1
		AJ1.14	AJ1.6	***Niigata/167/2010***, Tochigi/108/2008	2

Table 1. Continued

COI			16S rRNA Haplotype[c]	Sample name (Prefecture/ID/Year)	Sample number
Haplogroup[a]	Sub-haplogroup	Haplotype[b]			
A	AJ2	AJ2.1	AJ2.1	**Hyogo/A/2010**, **Hyogo/B/2010**, **Hyogo/C/2010**, **Hyogo/D/2010**, Hyogo/152/2009, Hyogo/153/2009, **Okayama/178/2010**, **Okayama/179/2010**, Okayama/180/2010, **Okayama/187/2010**	10
			AJ2.2	Hyogo/E/2010	1
		AJ2.2	AJ2.4	***Okayama/186/2010***	1
B	BJ1	BJ1.1	BJ1.1	Akita/192/2010	1
			BJ1.3	**Gifu/P10-2116/2010**	1
		BJ1.2	BJ1.1	**Hokkaido/177/2010**, ***Iwate/168/2010***	2
		BJ1.3	BJ1.1	***Mie/200/2010***	1
		BJ1.4	BJ1.1	Ibaraki/150/2009	1
		BJ1.5	BJ1.1	Shizuoka/29/2004	1
		BJ1.6	BJ1.1	Chiba/205/2011	1
	BJ2	BJ2.1	BJ2.1	**Ehime/169/2010**, Gifu/194/2010, **Hyogo/166/2010**, Kagawa/14/2003, Kagawa/125/2008, Kagoshima/26/2004, Mie/154/2009, Mie/170/2010, Miyazaki/141/2009, **Nagasaki/P10-2140/2010**, Nagasaki/P10-2028/2010, **Nara/203/2011**, Oita/P10-2202/2010, Tokushima/174/2010	14
			BJ2.3	Aichi/233/2011	1
			BJ2.5	***Ibaraki/188/2010***	1
		BJ2.2	BJ2.1	Mie/201/2010	1
		BJ2.3	BJ2.1	Aichi/234/2011	1
		BJ2.4	BJ2.6	***Oita/H1/2010***	1
		BJ2.5	BJ2.1	Hiroshima/124/2008	1
		BJ2.6	BJ2.1	Kagawa/126/2008, Kumamoto/101/2008	2

Table 1. Continued

COI			16S rRNA Haplotype[c)]	Sample name (Prefecture/ID/Year)	Sample number
Haplogroup[a)]	Sub-haplogroup	Haplotype[b)]			
ND	ND	ND	AJ1.1	Aichi/151/2009, Aichi/258/2012, Aichi/259/2012, Akita/56/2006, Chiba/8/2003, Chiba/17/2004, Chiba/34/2004, Chiba/35/2004, Chiba/42/2005, Chiba/50/2005, Chiba/54/2006, Chiba/57/2006, Chiba/62/2006, Chiba/64/2006, Chiba/70/2007, Chiba/87/2007, Chiba/107/2008, Chiba/115/2008, Chiba/116/2008, Chiba/131/2009, Chiba/138/2009, Chiba/139/2009, Chiba/162/2010, Chiba/212/2011, Chiba/217/2011, Chiba/238/2011, Chiba/239/2011, Chiba/240/2011, Chiba/252/2012, Chiba/271/2012, Chiba/274/2012, Chiba/219H/2011, Chiba/220H/2011, Chiba/P10-1715/2010, Ehime/237/2011, Fukui/232H/2012, Fukushima/32/2004, Gifu/53/2006, Gifu/123/2008, Gifu/122H/2009, Gunma/72/2007, Gunma/83/2007, Gunma/94/2007, Gunma/118/2008, Gunma/135/2009, Gunma/209/2011, Hiroshima/257/2012, Hyogo/216/2011, Ibaraki/41/2005, Ibaraki/45/2005, Ibaraki/49/2005,Ibaraki/74/2007, Ibaraki/80/2007, Ibaraki/92/2007, Ibaraki/110/2008, Ibaraki/222/2011, Ibaraki/267/2012, Ishikawa/65/2006, Iwate/236/2011, Kagoshima/142/2009, Kanagawa/16/2004, Kanagawa/157/2009, Kyoto/160/2010, Mie/11/2003, Mie/208/2011, Miyagi/79/2007, Miyazaki/249/2012, Nagasaki/277/2012, Niigata/250/2012, Saitama/68/2007, Saitama/129/2009, Shizuoka/76/2007, Tochigi/84/2007, Tochigi/86/2007, Tochigi/104/2008, Tochigi/146/2009, Wakayama/245/2011, Yamaguchi/78/2007, Yamanashi/100/2008, Yamanashi/120/2008, Yamanashi/256/2012	81
			AJ1.2	Chiba/251/2012, Saitama/235/2011	2
			AJ1.4	Tochigi/77/2007	1
			AJ1.6	Niigata/112/2008	1
			AJ1.7	Saitama/271H/2012	1
			AJ1.8	Mie/219/2011	1
			AJ1.9	Saitama/264/2012	1
			AJ1.11	Iwate/220/2011, Kanagawa/23/2004, Saitama/117/2008	3
			AJ2.1	Okayama/231/2011, Okayama/275/2012	2
			AJ2.3	Hyogo/161/2010	1
			AJ2.5	Okayama/232/2011	1

Table. 1. Continued

COI			16S rRNA Haplotype[c)]	Sample name (Prefecture/ID/Year)	Sample number
Haplogroup[a)]	Sub-haplogroup	Haplotype[b)]			
ND	ND	ND	BJ1.1	Aomori/218H/2011, Chiba/206/2011, Chiba/207/2011, Chiba/210/2011,Chiba/230/2011,Chiba/244/2011, Chiba/253/2012, Chiba/269/2012, Chiba/254H/2012, Hokkaido/51/2005, Hyogo/213/2011, Ibaraki/267H/2002, Ishikawa/229/2011, Iwate/22/2004, Iwate/46/2005, Iwate/242/2011, Nagano/272H/2012, Saga/218/2011, Tochigi/97/2007	19
			BJ1.2	Ehime/226/2011, Ehime/228/2011	2
			BJ2.1	Chiba/119/2008, **Chiba/175/2010**, ***Chiba/189/2010***, Chiba/254/2012, Chiba/272/2012, Ehime/223/2011, Ehime/225/2011, Ehime/227/2011, Hyogo/128/2008, Hyogo/248/2012, Ibaraki/214/2011, Kyoto/12/2003, Mie/241/2011, Mie/137/2009, Mie/215/2011, Miyazaki/145/2009, Nagasaki/268/2012, Tottori/123H/2010, Wakayama/246/2011, Yamaguchi/85/2007	20
			BJ2.2	Tokushima/136/2009	1
			BJ2.4	Shizuoka/243/2011	1
Total		28 haplotypes	26 haplotypes		239

ND: not determined

a) Haplogroups reported by Oines and Brannstrom [35]

b) Haplotypes were determined based on the nucleotides sequences of 643 bp of COI gene.

c) Haplotypes were determined based on the nucleotides sequences of 397 bp of 16S rRNA

Sample names which have type I and II ITS sequences are shown in bold face and italicized bold face, respectively.

Fig. 1.

Fig. 1. Phylogenetic tree constructed with nucleotide sequences of COI gene using Neighbour-Joining method. The tree was constructed with 476-bp of COI gene sequences of *D. gallinae* in Japan determined in this study and those of other countries obtained from the database. Haplotypes designated based on the 643-bp of COI gene of 101 mite samples collected in Japan are shown in boldface. The haplotypes previously reported [35] in other countries are underlined. Other sequences obtained from the database are shown with the accession number and the country where the mites were detected. Bootstrap values more than 700 are shown. The haplotypes, A, B, C and D, previously reported [35] are indicated with vertical thick lines. The haplotypes found in Japan clustered in haplogroups A and B. The haplotypes found in Japan in each haplogroup A and B diverged into two clades designated as sub-haplogroups AJ1 and AJ2, and BJ1 and BJ2, respectively. These sub-haplogroups are indicated with thin vertical lines. Sub-haplogroups AJ2 and BJ1 are closely related to the haplotypes found in European countries. However, the haplotypes in sub-haplogroups AJ1 and BJ2 are relatively distant from the haplotypes found in other countries.

Fig. 2.

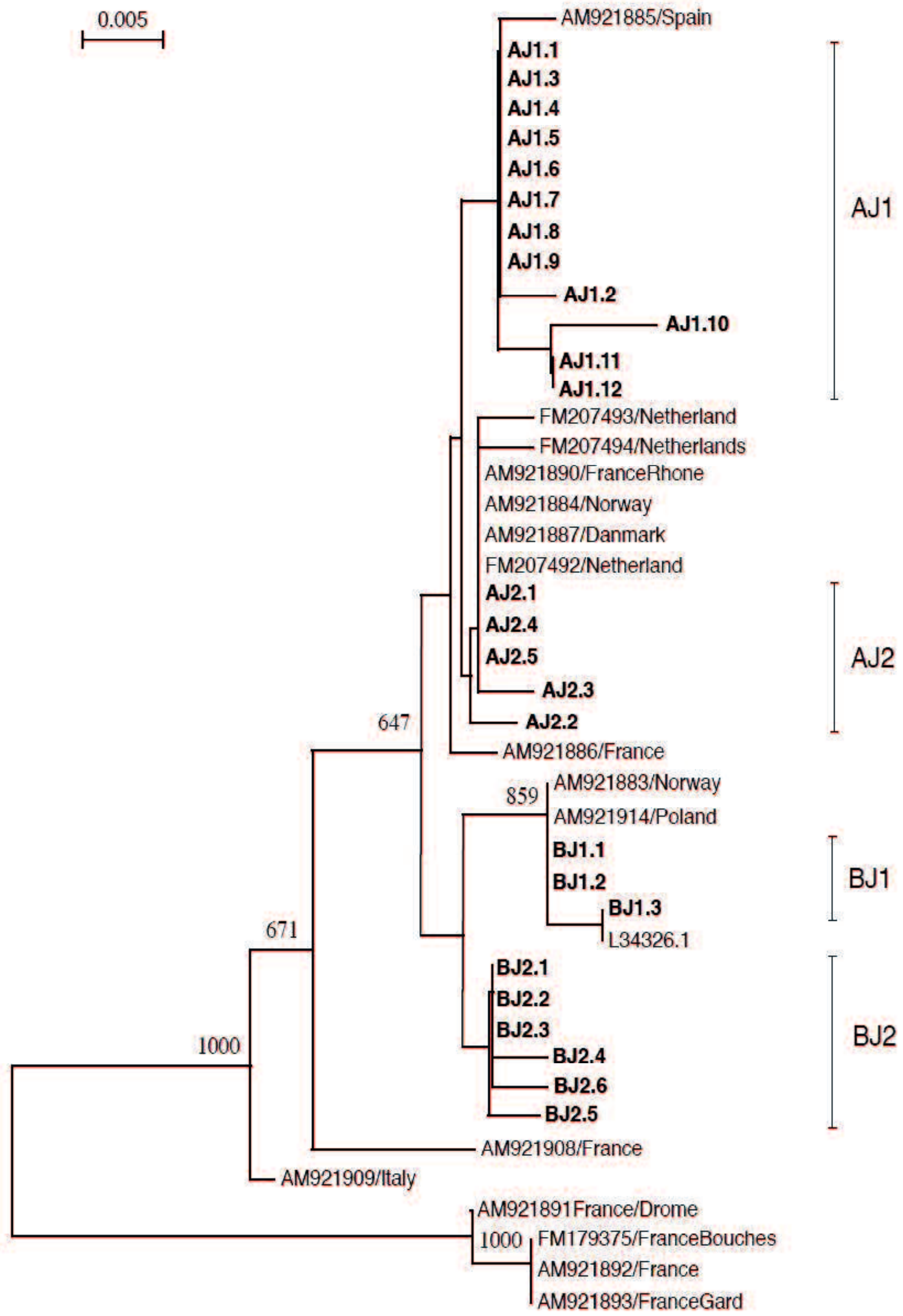

Fig. 2. Phylogenetic tree constructed with the nucleotide sequences of 16S rRNA gene using Neighbor-Joining method. The tree was constructed with 299-bp of 16S rRNA gene sequences of *D. gallinae* in Japan determined in this study and those of other countries obtained from the database. Haplotypes found in Japan were designated based on the 397-bp of 16S rRNA gene of *D. gallinae* obtained from 239 samples and are shown in boldface in the tree. Other sequences obtained from the database are shown with the accession number and the country where the mites were detected. Bootstrap values more than 600 are shown. The haplotypes found in Japan clustered in two haplogroups. Subsequently, the haplotypes in each haplogroup diverged into two lineages designated as sub-haplogroups, AJ1 and AJ2, and BJ1 and BJ2, respectively. These sub-haplogroups are indicated with the thin vertical lines. Sub-haplogroups AJ2 and BJ1 are closely related to the sequences found in European countries. However, the sub-haplogroups AJ1 and BJ2 are relatively distant from the haplotypes found in other countries.

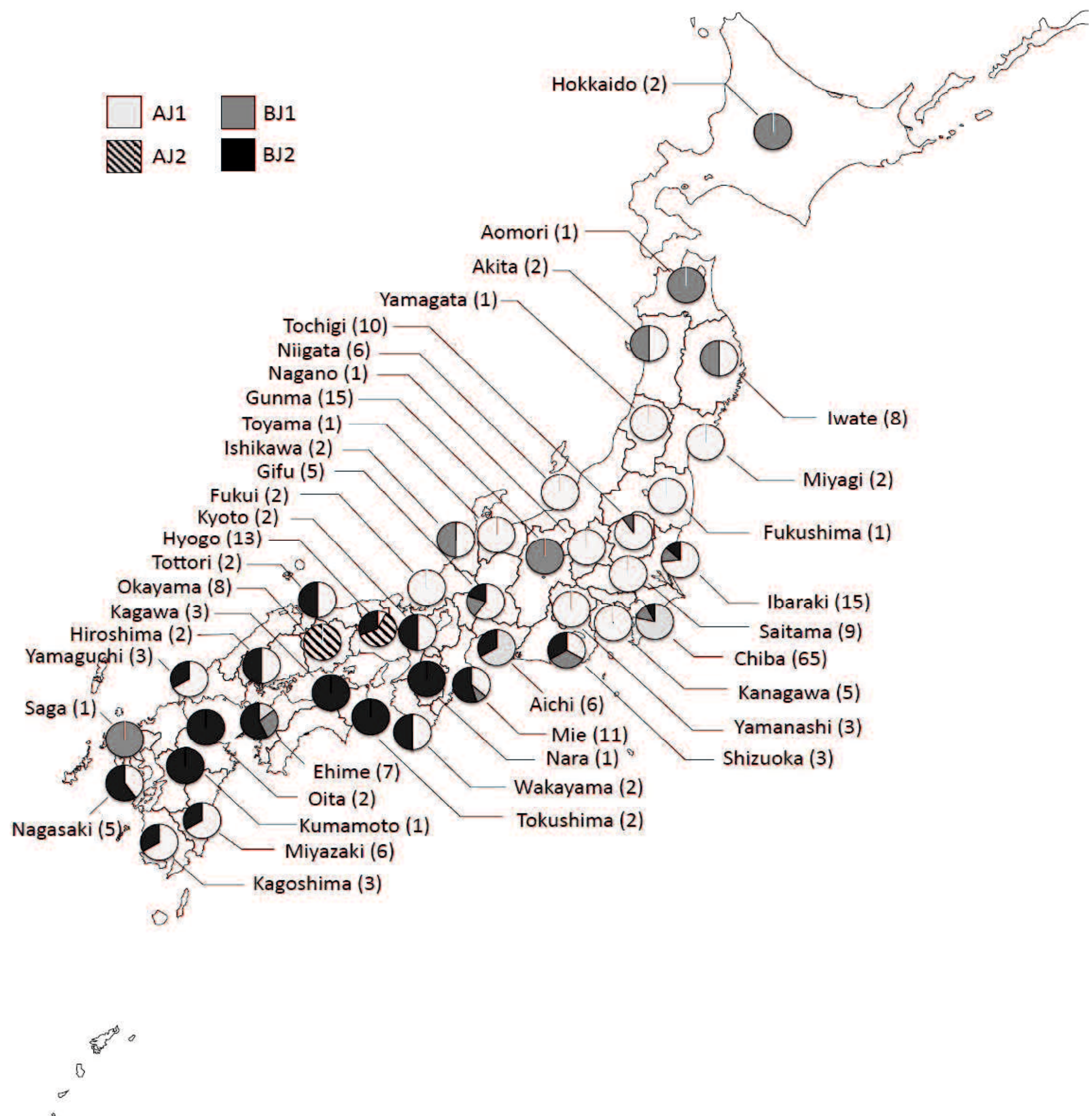

Fig. 3. Geographical distribution of sub-haplogroups of *D. gallinae* in Japan based on 16S rRNA sequences. Proportions of 4 sub-haplotypes, AJ1, AJ2, BJ1 and BJ2, found in 40 prefectures are shown in pie charts on map. The prefecture names are shown with sample numbers in brackets. The sub-haplogroups AJ1 and BJ1 were found all over Japan. AJ2 was found only in two adjacent prefectures, Hyogo and Okayama. BJ2 was mainly distributed in the western area of Chiba prefecture.

CHAPTER 2

Molecular detection of avian pathogens in poultry red mite, *Dermanyssus gallinae,* collected in chicken farms

Introduction

Dermanyssus gallinae (Acari, Mesostigmata, Dermanyssoidea, Dermanyssidae), poultry red mite (PRM), is an obligatory blood-sucking parasite of both domestic and wild birds. The mite has been reported to parasitize at least 28 avian species [35] and is considered one of the major problems in poultry farms in the United States [15], Europe [6] and Japan [34]. The infestation of PRM in poultry results in stress, decreasing egg production, anemia, and even mortality due to exsanguination.

PRMs carry and are potential vectors of several pathogens including *Salmonella enterica* (SE) [20, 57], *Erysipelothrix rhusiopathiae* (ER) [8] and avian pox virus (APV) [7, 48]. The mites can also be sites of *Salmonella* Enteritidis multiplication [57]. However, the prevalence of avian microbial pathogens in PRM over a wide region has not been studied.

In the present study, to define the role of PRM in transmitting and maintaining avian pathogens in poultry farms, PRMs were collected from chicken farms throughout Japan and examined for DNAs of APV, ER and SE. We also examined PRM for the presence of four other pathogens that are frequently detected in chicken farms but that so far have not been detected in PRM: *Mycoplasma synoviae* (MS), *Mycoplasma gallisepticum* (MG), fowl Adenovirus (FAdV) and Marek's disease virus (MDV).

Materials and Methods

Dermanyssus gallinae

A total of 159 samples (at least 50 for each sample) were obtained from 142 chicken farms in 38 prefectures throughout Japan from 2004 to 2012. In all of the farms, the chickens appeared healthy. Each sample was stored in 99.5% ethanol at room temperature until use.

DNA preparation

Mite DNA samples used in this study were obtained from 10 pooled adult mites. The mites were homogenized with zirconia beads using TissueLyser II (Qiagen Inc, Chatsworth, CA, U. S. A.) in 20 μl of buffer 1 provided by a Ten Minute DNA Release Kit -1 (Jacksun Easy Biotech Inc., U. S. A.), and the DNA samples were prepared according to the manufacturer's instructions. When a sample was found to be positive for one of the seven pathogens, another DNA sample was prepared as described below to estimate whether the pathogens exist on surface or internal side of the PRM. Ten mites prepared from the sample that was positive for any pathogens were washed out before the DNA preparation to remove the microorganisms on the surface of the mites as previously described [18]. Briefly, ten mites taken from the 99.5% ethanol were rinsed three times in 500 μl of sterile ultrapure water with vigorous shaking. Then the washed mites were used for DNA preparation. If the PCR using the DNA sample prepared from the washed mites was positive, it was speculated that the pathogen existed internal side of PRM. DNA

samples of commercial vaccines were also prepared with the same DNA extraction kit as a control. The DNA samples prepared were stored at -30°C until use.

PCR for detection of avian pathogens

The DNA samples were screened for seven avian pathogens including ER, MS, MG, SE, APV, FAdV and MDV by PCR as described below. The target genes and their PCR primers are shown in Table 2.

For detection of APV DNA, primers P1x and P2 were used to amplify part of the 4b core protein gene [25]. Primer P1x was designed based on the sequence of primer P1 [25] by adding one more T nucleotide to the 5' end to improve the sensitivity (our unpublished data). If the gene coding for the 4b core protein was detected, the viral DNA was further examined to determine whether it was derived from the wild type APV or commercial vaccine strain. Because the wild type APV genome includes an intact reticuloendotheliosis (RE) provirus and the genome of the vaccine virus includes only the truncated LTR sequence of RE provirus [14], to detect the insertion of intact RE provirus sequence in APV genome, PCR using heterologous primer set that anneal to *env* gene of RE provirus (REVenv7F primer) [14] and FPV ORF 203 (FPV203 3R primer) [14] was conducted as semi-nested first PCR. When this PCR was negative, semi-nested second PCR using primers REVenv7F and TR2 [49] that anneals to the internal region flanking to FPV203 3R primer annealing site was conducted. When the first or second PCRs were positive, we concluded that intact RE provirus insertion, that was specifically found in wild type APV, was positive. Each primer sequence, target genes and expected length of the PCR products were shown in

Table 2. DNAs prepared from the commercial vaccines used in Japan were used as negative controls for the presence of RE provirus.

Primers MSL1 and MSL2 [30] (Table 2) were used to detect the MS 16S rRNA sequence. Subsequently, the nucleotide sequences of PCR products were compared with the sequence derived from the MS-H vaccine strain used in Japan to differentiate between the MS wild type and the vaccine strain [5].

Primers Mgc2 2F and Mgc2 2R were used to detect the MG *mgc2* gene [13]. The obtained nucleotide sequences were used to differentiate MG from 5 commercial live vaccine strains used in Japan (ts-11, 6/85, MGS, G210 and K5831-B19) following a previous report [27].

PCRs were performed in a total volume of 25 μl containing 2 μl of DNA template (or 0.5 μl of the first PCR product in semi-nested second PCR) prepared as described above, 1 μl of forward and reverse primer mixture (final concentration was 0.5 μM each) and 12.5 μl of GoTaq Green Master Mix, 2X (Promega, Madison, WI, U. S. A.). The PCR conditions for each pair of primers were employed according to the previous reports listed in Table 2. Amplification products were confirmed by electrophoresis using 1.2% (w/v) agarose gels, stained with 0.1% Gel Red (Biotium, Inc., Hayward, CA, U. S. A.) and visualized with a UV transilluminator. To confirm the specificity of the PCR, nucleotide sequence of the PCR products was determined by Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U. S. A.) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, U. S. A.).

Results

Detection of avian pathogens in *D. gallinae*

Twenty-five (15.7%) of the 159 DNA samples prepared from the mites without washing prior to the DNA preparation were positive for a single pathogen, either APV or MS or MG while the DNAs of ER, SE, FAdV and MDV were not detected (Table 3). Fifteen of these 25 samples were positive only for APV, 9 samples and 1 sample were positive only for MS and MG, respectively. In addition, seven samples (4.4%) without washing were multiple pathogens positive. Of the seven DNA samples, six were positive for both APV and MS, and another was positive for both APV and MG. In total, 32 (20.1%) of the 159 DNA samples, 25 for single pathogen and 7 for multiple pathogens, were positive in PCRs conducted in this study. APV, MS and MG positive samples were detected from 12, 12 and 2 prefectures, respectively. Apparent geographical bias was not found in distribution of the positive samples.

Avian pox virus

The APV gene was totally detected in 22 of the 159 PRM DNA samples (13.8%) prepared from the mites without washing. Furthermore, 4 of the 22 DNA samples prepared from the mites after washing were also positive for APV. Nineteen samples positive for APV were also positive in PCR for REV *env* to FPV ORF 203 region.

M. synoviae

In total, fifteen of the 159 DNA samples (9.4%) prepared from the mite without washing were positive for the MS 16S rRNA. In addition, two of the 15 DNA samples prepared from the mite after washing were positive for the rRNA.

Of the fifteen positive samples, seven of these sequences were identical to the sequence of the vaccine MS-H used in Japan. The eight other sequences differed by at least 1 nucleotide from the sequence of MS-H vaccine. Two of these 8 sequences were identical to each other and differed by 2 nucleotides from the MS-H sequence. These two sequences were detected from different chicken houses in the same farm.

M. gallisepticum

Two of the 159 DNA samples (1.3%) prepared from the unwashed mites were positive for the MG *mgc2* gene (Table 3). However, when the two DNA samples were prepared from the washed mites, no DNA samples were positive for the *mgc2* gene. Of the two positive DNA samples, one sequence was identical to the sequence of vaccine strain G210 and the other was identical to the sequence of vaccine strain K5831-B19.

Detection of multiple pathogens

Of the six samples positive for both APV and MS, 5 samples were positive in PCR for REV *env* to FPV ORF 203 region. In addition, MS 16S rRNA sequences detected from 3 of these 5 samples were different from the sequence of MS-H vaccine.

In the case of another multiple pathogens positive sample, which was positive for both APV and MG, PCR for REV *env* to FPV ORF 203 region was positive and the sequence of MG *mgc2* gene detected were identical to the sequence of vaccine strain K5831-B19.

Discussion

In the present study, APV, MS and MG DNAs were detected in 22, 15 and 2 DNA samples prepared from the unwashed mites, respectively. Of the total, 25 samples were single pathogen positive and 7 samples were multiple pathogens positive. These results indicate the possibility that the PRM can transfer APV, MS and MG. In addition, 4 and 2 of 32 DNA samples prepared from the mites after washing were positive for APV and MS, respectively. Although the positive number decreased after washing, APV or MS was detected even after 3 times washing in the mites. Because the washing step is expected to remove microorganisms on the surface of the mites, this result implied that PRM can harbor APV and MS both externally and internally.

Of the 22 DNA samples positive for APV, 19 were also positive for REV *env* to FPV ORF 203 region. This finding shows that the 19 samples have intact RE provirus reported as a virulence marker for wild-type APV [50]. In Japan, almost all chickens are vaccinated for APV infection and only a few outbreaks of avian pox are reported each year. However, this finding indicates that wild-type APV is latently distributed even in apparently healthy farms and that PRM may play a role in transmission of the virus in chicken farms. In fact, outbreaks of avian pox were reported even in vaccinated chicken flocks in farms where the mites were present [7]. Because PRM is known as a mechanical vector of APV [48], in addition to vaccination against APV infection, eradiation of PRM is needed for effective prevention of the disease.

Of the 15 MS 16S rRNA sequences obtained in this study, eight were different from the MS vaccine sequence, indicating that the MS detected was the wild type (Table 3). This shows that PRM can harbor wild-type MS, which is a possible chicken pathogen. Although this finding raises the possibility that PRM can transmit MS among chickens,

further studies such as experimental infection of MS to chicken through a PRM may be needed to confirm it. Moreover, the finding of two identical wild-type MS sequences in different chicken houses in the same farm implied that PRM can spread MS among chicken houses in a farm. To our knowledge, this is the first report to show the presence of a mycoplasma in PRM.

Of the six mite samples positive for both APV and MS, three samples were positive in PCR for REV *env* to FPV ORF 203 region, and the MS 16S rRNA sequences detected were different from the sequence of vaccine strain. These results indicate that both of the APV and MS detected from the 3 samples were wild-type (Table 3). These findings suggest that the mite can harbor and transmit more than one avian pathogen in poultry farms. This is the first report to show the presence of multiple pathogens in PRMs.

In European countries, ER was found in PRM collected from infected farms [8] and SE was found in mites collected from apparently healthy farms [20]. The infections of SE and ER among some chickens have been reported in Japan [46, 55]. However, these bacteria were not detected in any of the present mite samples. Furthermore, although FAdV and MDV are ubiquitous pathogens in chicken farms [32, 39], they were not detected in any samples in this study. Although the reason why these pathogens were not detected in this study was not clear, because the PRM samples used in this study were stored in ethanol until use, some organisms could be lysed by ethanol and it may affect the results. Alternatively, PRMs may be rarely associated with the spread of these pathogens in Japan. To define the actual distribution of these organisms in PRM, further studies using PRM without fixation may be needed.

This study demonstrated that APV, MS and MG including wild type strains are prevalent in PRMs. Although further studies about the actual transmission activity of the mites for these pathogens are needed, these findings suggest that PRM may transfer these pathogens among chickens. The present results suggest that eradication of PRM will not only reduce the negative effects of blood-sucking but also decrease the transmission of these pathogens in poultry farms.

Table 2. List of primers used for detection of each pathogen

Pathogens	Primers[a)]	Primer sequences	Target sequences	Length of PCR products	References
Erysipelothrix rhusiopathiae	ER1F ER1R	5'-gttcatctctctaatgcactac-3' 5'-tgttggactactaatcgtttcg-3'	23S rRNA	399 bp	[56]
Mycoplasma gallisepticum	Mgc2 2F Mgc2 2R	5'-cgcaatttggtcctaatccccaac-3' 5'-taaacccacctccagctttatttc-3'	*mgc2*	236-302 bp	[13]
Mycoplasma synoviae	MSL1 MSL2	5'-gagaagcaaaatagtgatatca-3' 5'-cagtcgtctccgaagttaacaa-3'	16S rRNA	211 bp	[30]
Salmonella enterica	Stn – 101 Stn – 111	5'-ctttggtcgtaaaataaggcg-3' 5'-tgcccaaagcagagagattc-3'	*enterotoxin*	260 bp	[61]
	16S F 16S R	5'-tgttgtggttaataaccgca-3' 5'-cacaaatccatctctgga- 3'	16S rRNA	574 bp	[61]
Avipox virus	P1x P2	5'-tcagcaggtgctaaacaaca-3' 5'-cggtagcttaacgccgaata-3'	4b core protein	579 bp	In this study [25]
	REVenv7F FPV203 3R	5'-cctgactcgcattatccatgacaa-3' 5'-ttcaaccaccaggctacataaagg-3'	REV *env* to FPV ORF203 for the semi-nested first PCR	740 bp	[14]
	REVenv7F TR2	5'-cctgactcgcattatccatgacaa-3' 5'-cacacgaatataccaataagg-3'	REV *env* to FPV ORF203 for the semi-nested second PCR	579 bp	[14] [49]
Fowl Adenovirus	Hex L1-s Hex L1-as	5'-atgggagcSacctaYttcgacat-3' 5'- aaattgtcccKRaaNccgatgta-3'	*hexon*	590 bp	[54]
Marek's disease virus	MEQ FP MEQ RP	5'-ggatcgcccaccacgattactacc-3' 5'-actgcctcacacaacctcatctcc-3'	*meq*	400 bp	[39]

a) Forward and reverse primers

Table 3. Sample numbers and the distribution of the mite samples which showed positive in PCR for detection of avian pathogen DNAs from the mite samples without washing

Prefectures	Sample number	Single pathogen positive							Multiple pathogens positive					All negative (%)
		APV[a)]		MS[b)]		MG[c)]		Subtotal (%)	APV Wild & MS Vac (%)	APV Wild & MS Wild (%)	APV Vac & MS Vac (%)	APV Wild & MG Vac (%)	Subtotal (%)	
		Wild (%)	Vac[d)] (%)	Wild (%)	Vac (%)	Wild (%)	Vac (%)							
Akita	1	1 (100)	0	0	0	0	0	1 (100)	0	0	0	0	0	0
Aomori	2	0	0	1 (50.0)	0	0	0	1 (50.0)	0	0	0	0	0	1 (50.0)
Chiba	44	5 (11.4)	0	1 (2.3)	0	0	1 (2.3)	7 (15.9)	1 (2.3)	0	0	0	1 (2.3)	36 (81.8)
Fukui	2	0	0	0	0	0	0	0	0	1 (50.0)	0	0	1 (50.0)	1 (50.0)
Gunma	6	1 (16.7)	0	0	0	0	0	1 (16.7)	0	0	0	0	0	5 (83.3)
Hokkaido	1	0	0	0	0	0	0	0	0	1 (100)	0	0	1 (100)	0
Hyogo	11	2 (18.2)	0	1 (9.1)	0	0	0	3 (27.3)	0	1 (9.1)	0	0	1 (9.1)	7 (63.6)
Ibaraki	6	1 (16.7)	0	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	1 (16.7)	4 (66.7)
Iwate	7	0	0	1 (14.3)	0	0	0	1 (14.3)	0	0	0	0	0	6 (85.7)
Kanagawa	3	1 (33.3)	0	1 (33.3)	1 (33.3)	0	0	3 (100)	0	0	0	0	0	0
Mie	12	1 (8.3)	0	0	0	0	0	2 (16.7)	0	0	1 (8.3)	1 (8.3)	2 (16.7)	9 (75.0)
Nagasaki	6	0	1 (16.7)	0	1 (16.7)	0	0	2 (33.3)	0	0	0	0	0	4 (66.7)
Okayama	8	0	1 (12.5)	0	1 (12.5)	0	0	2 (25.0)	0	0	0	0	0	6 (75.0)
Shizuoka	3	1 (33.3)	0	0	0	0	0	1 (33.3)	0	0	0	0	0	2 (66.7)
Tochigi	3	0	0	0	1 (33.3)	0	0	1 (33.3)	0	0	0	0	0	2 (66.7)
Other 23 prefectures[e)]	44	0	0	0	0	0	0	0	0	0	0	0	0	44 (100)
Total	159	13 (8.2)	2 (1.3)	5 (3.1)	4 (2.5)	0	1 (0.6)	25 (15.7)	2 (1.3)	3 (1.9)	1 (0.6)	1 (0.6)	7 (4.4)	127 (79.9)

Erysipelothrix rhusiopathiae, *Salmonella enterica,* fowl Adenovirus and Marek's disease virus were not detected.

a) If the intact RE provirus integration was detected, such samples were considered as wild type. If the intact RE provirus integration was not detected, such samples were considered as vaccine.

b) If 16S rRNA detected was different from the sequence of vaccine used in Japan, such samples were considered as wild type. If 16S rRNA detected was identical to the sequence of vaccine used in Japan, such samples were considered as vaccine.

c) If mgc2 sequence detected was different from the sequence of vaccine used in Japan, such samples were considered as wild type. If mgc2 sequence detected was identical to the sequence of vaccine used in Japan, such samples were considered as vaccine.

d) Vac: vaccine

e) Other 23 prefectures: Aichi, Ehime, Fukushima, Gifu, Hiroshima, Ishikawa, Kagoshima, Kumamoto, Kyoto, Miyagi, Miyazaki, Nara, Niigata, Oita, Saga, Saitama, Tokushima, Tottori, Toyama, Wakayama, Yamagata, Yamaguchi and Yamanashi.

GENERAL CONCLUSIONS

Dermanyssus gallinae is an obligatory blood-sucking ectoparasite as well as a possible vector of several avian pathogens. Genetic diversity of the mite distributed in European countries indicated the possibility of internal transmission of the mite among different countries. Although the mites are distributed throughout the world, such a phylogenetic study has not been conducted in Asian countries. Moreover, a comprehensive study about the distribution of avian pathogens in PRM has not been conducted.

In this thesis, a part of the mitochondrial cytochrome *c* oxidase subunit I (COI), 16S rRNA genes and nuclear internal transcribed spacers (ITS) region in 239 mite samples collected from 40 prefectures throughout Japan were sequenced. The COI and 16S rRNA nucleotide sequences were classified into 28 and 26 haplotypes, respectively. In phylogenetic analyses, the haplotypes clustered into 2 haplogroups corresponding to haplogroups A and B, which were previously reported. Haplogroups A and B were further subdivided into sub-haplogroups AJ1 and AJ2, and BJ1 and BJ2, respectively. In both trees, the sequences of haplotypes in AJ1 and BJ2 were relatively distant from those reported in other countries, while some sequences in AJ2 and BJ1 were identical to those in Europe. In addition, the ITS sequences were classified into two sequences, and both sequences were closely related to the sequences found in European countries. These findings indicate a possibility of international overseas transmission of *D. gallinae*.

In addition, a total of 159 PRM samples collected between 2004 and 2012 from 142 chicken farms in 38 prefectures in Japan were examined to define the role of PRM in the prevalence of avian infectious agents. Polymerase chain reactions (PCR) for

seven pathogens: avian pox virus (APV), *Erysipelothrix rhusiopathiae* (ER), *Salmonella enterica* (SE), *Mycoplasma synoviae* (MS), *Mycoplasma gallisepticum* (MG), fowl Adenovirus (FAdV) and Marek's disease virus (MDV) were conducted. APV DNA was detected in 22 samples (13.8%), 19 of which were wild-type APV. 16S rRNA of MS was detected in 15 samples (9.4%) and the *mgc2* gene of MG was detected in 2 samples (1.3%). The DNAs of ER, SE, FAdV and MDV were not detected in any samples. The sequences of eight of 15 MS 16S rRNA sequences detected were different from the vaccine sequence, indicating they were wild-type strains, while both of the MG *mgc2* gene sequences detected were identical to the vaccine sequences. Of these avian pathogen-positive mite samples, six were positive for both APV and MS. Three of the 22 APV-positive mite samples remained positive after being washed, indicating that the mites can harbor the pathogen internally. These findings indicated that PRM can harbor the wild-type pathogens and might play a role as a vector in spreading these diseases in farms.

The present results indicate the possibility of overseas transmission of *D. gallinae*. In addition, the detection of wild-type APV and MS DNAs in the mites raises the possibility that PRM contributes to the spread of avian pathogens worldwide. For a comprehensive understanding of role of *D. gallinae* in distributing infectious diseases around the world, further worldwide epidemiological studies will be needed.

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ABSTRACT

Dermanyssus gallinae, poultry red mite (PRM), was first described by De Geer in 1778. It is a blood-feeding arthropod parasite belonging to the subclass Acari, superorder parasitiformes, order Mesostigmata (Gamasida), in the family Dermanyssidae. PRM is distributed throughout the world including Europe, Africa, North and South America, Australia and Asia including Japan. It is a significant pest to laying hens. It is less than 1.5mm in length and can rapidly propagate in poultry houses. It causes a decline in egg quality and egg production, anemia and even death. It is also a potential vector of infectious diseases. Phylogenetic analyses of European PRMs suggest that they are transmitted among different countries, raising the possibility that some pathogens may be spread with them. Similar phylogenetic studies, which are essential for epidemiological studies, have not been conducted in Asian countries. In addition, a comprehensive study of the distribution of avian pathogens in PRM has not been conducted. In the present study, the phylogenetic and genetic diversity of mites distributed in Japan and other countries were examined. Furthermore, detection of several avian pathogens from the mite was attempted to assess the risk of transmission of infectious diseases by the mites.

To define the genetic diversities of the mite in Japan, 239 mite samples were collected during 2005 to 2012 from 40 prefectures throughout Japan. The nucleotide sequences of a part of the mitochondrial cytochrome oxidase subunit I (COI) and16S rRNA genes and nuclear internal transcribed spacers (ITS) region obtained from these samples were determined. The COI and 16S rRNA sequences were classified into 28 and 26 haplotypes, respectively. In phylogenetic trees, the haplotypes clustered into 2 haplogroups corresponding to haplogroups A and B, which were previously reported. Haplogroups A and B were further subdivided into sub-

haplogroups AJ1 and AJ2, and BJ1 and BJ2, respectively. In both trees, the sequences of haplotypes in AJ1 and BJ2 were relatively distant from those reported in other countries, while some sequences in AJ2 and BJ1 were identical to those in Europe. The ITS sequences were classified into two sequences, and both sequences were closely related to sequences found in European countries. Although the ITS sequences of some samples were different from each other, their haplotypes were the same. These results show that *D. gallinae* in Japan were genetically diverse, and raise the possibility of overseas transmission of *D. gallinae* between Europe and Japan. In addition, detection of different sequences of nuclear ITS region from the mites classified into same haplotype based on the mitochondrial genes suggests that a hybridization event occurred between different haplotypes of the mites in Japan.

To define the prevalence of avian infectious agents in the mite, 159 mite samples collected from 2004 to 2012 from 142 chicken farms in 38 prefectures were examined. Polymerase chain reactions (PCR) were conducted to check for the presence of seven pathogens (avian pox virus (APV), *Erysipelothrix rhusiopathiae* (ER), *Salmonella enterica* (SE), *Mycoplasma synoviae* (MS), *Mycoplasma gallisepticum* (MG), fowl Adenovirus (FAdV) and Marek's disease virus (MDV)). Because live vaccines against APV, MS and MG were used in Japan, additional PCR or sequence analyses were done to distinguish wild-type pathogens from the live vaccines. Of the 159 PRM samples, APV DNA was detected in 22 samples (13.8%), 19 of which were wild-type APV. Furthermore, MS DNA was detected in 15 samples (9.4%) and the MG DNA was detected in 2 samples (1.3%). Eight of 15 MS 16S rRNA sequences differed from the vaccine sequence, indicating they were wild-type strains, while both of the MG gene sequences detected were identical to the vaccine sequences. Of these avian pathogen-positive mite samples, six were positive for both

APV and MS. Four of the 22 APV-positive mite samples and 2 of the 15 MS-positive mite samples remained positive after being washed, indicating that the mites can harbor the pathogens internally. The DNAs of ER, SE, FAdV and MDV were not detected in any samples. These findings indicate that *D. gallinae* in Japan is contaminated with wild-type APV and MS and might play a role as a vector in spreading these diseases in farms.

The present results indicate the possibility of overseas transmission of *D. gallinae.* In addition, wild-type APV and MS DNAs were detected in the mite in Japan. Although the infectivities of these pathogens detected in the mites were not confirmed, these findings imply that PRM contributes to the spread of avian pathogens worldwide. For a comprehensive understanding about a role of *D. gallinae* in distributing infectious diseases around the world, further worldwide epidemiological studies will be needed.