Surveys of Viruliferous Alate Aphid of *Plum pox virus* in *Prunus mume* Orchards in Japan

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Abstract

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Plum pox virus (PPV) is transmitted by infected buds and aphids. It is important to analyze the outbreak trends and viruliferous rate of aphids in areas where the occurrence of PPV is reported, so as to develop strategies for disease control. Between April 2011 and December 2012, yellow insect-trapping adhesive plates were placed for 2 days at a time each week in an area where PPV is occurring in Japan. Outbreak trends were analyzed based on the trapped alate aphid samples, and up to 50 of them were tested per week to identify species and determine the rate of viruliferous specimens. Although the number of aphids varied according to survey year, three peaks were noticeable in each year. Based on the sequence

data for the mitochondrial cytochrome c oxidase I region, approximately 40 different species of aphid were trapped in both years. Of the five dominant species of aphids identified during the 2 years, *Aphis spiraeicola* was trapped in large numbers. PPV-positive aphids were higher in fall onward, when the total number of trapped aphids decreased, than in spring and summer, when a larger number of aphids was caught. PPV transmission tests using the most abundant species revealed that *A. spiraeicola*, *A. craccivora*, *A. gossypii*, and *Rhopalosiphum maidis* were transmitters, although *A. spiraeicola* is likely of epidemiological significance.

Blossoms of the Japanese apricot (*Prunus mume* Sieb. et Zucc.) have been admired since ancient times in Japan and its fruit has been processed into *umeboshi* (salted apricot) or *umeshu* (apricot wine), making the tree commercially highly valuable. However, an outbreak of *Plum pox virus* (PPV) in Tokyo (Maejima et al. 2010) could potentially pose a serious threat to *P. mume* production in Japan.

PPV was confirmed for the first time in Bulgarian plum in 1915 (Atanasoff 1932). Since then, it has spread across nearly the entire region of Europe during the 1970s (Llacer and Cambra 1998; Roy and Smith 1994); coastal areas of the Mediterranean, including Egypt and Syria in the 1980s (Cambra et al. 2004; Kaya et al. 2014; Mazyad et al. 1992); and to India, China, Chile, and North America in the 1990s (Reyes et al. 2003; Rosales et al. 1998).

In accordance with the Plant Protection Act, PPV is designated as a pest subject to quarantine in Japan and caution is exercised to prevent invasion. The first case of a PPV (D strain) was confirmed in April 2009 on a plum tree in Ome City, Tokyo (Maejima et al. 2010). Since then, the infection on plum trees has rapidly spread throughout Japan and; in 2013, 11,593 infected trees were newly found in eight prefectures. In Ome City, which is famous for its Japanese apricot blossom viewing and where PPV was first detected in Japan, more than 26,000 plum trees have been cut down thus far to prevent the further spread of PPV. The areas where PPV infection was confirmed in 2013 included the Kansai region, which accounts for a dominant share of the domestic *P. mume* yields, and its potential negative

impacts on the industry and tourism are of major concern (Tsuda and Sano 2014).

PPV belongs to the genus *Potyvirus* in the family *Potyviridae*. Virions form filamentous particle, 660 to 770 nm in length and 12.5 to 20 nm in width. The virus comprises a positive-sense, single-stranded RNA genome, coding a P1 protein with a proteolytic activity, with a molecular mass of approximately 35 kDa; a helper-component proteinase (HC-Pro) involving aphid transmission, with a molecular mass of approximately 52 kDa; a P3 protein relating to symptom development, with a molecular mass of approximately 50 kDa; a cylindrical inclusion protein found in the cytoplasm, with a molecular mass of approximately 71 kDa; a viral genome-linked protein, with a molecular mass of approximately 21 kDa; an NIa-Pro with serine-like cysteine protease, with a molecular mass of approximately 27 kDa; an NIb with the RNA-dependent RNA polymerase activity, with a molecular mass of approximately 58 kDa; a coat protein, with a molecular mass of approximately 30 kDa; and three small open reading frames (ORF) of a pretty interesting potyvirus ORF, 6K1, and a 6K2, respectively (Fauquet et al. 2005). PPV is categorized into eight strains (D, M, Rec, C, CR, W, EA, and T) according to serological and molecular characteristics (Glaser et al. 2013), among which D, M, and Rec are the major strains worldwide. The D strain was first isolated from *P. armeniaca* in southern France and is spreading worldwide, including in the United States, Canada, and China. The M strain was isolated from *P. persica* in Greece and has been confirmed in many locations throughout Europe. In addition, the M strain has been reported to be more efficiently transmitted via aphid vectors than the D strain (Dallot et al. 1998). The Rec strain was isolated from *P. domestica* in Slovakia and results from recombination of the D and M strains (Glaser et al. 2002).

PPV is transmitted by grafting, sap, or insects but is not transmitted via seed. With the exception of dissemination through the use of symptomless infected budwood, the most prominent mode of transmission under natural conditions is insect transmission. PPV can be transmitted by more than 20 different types of aphids (Goytia et al. 2006). Aphids that have acquired PPV can transmit it only within a timeframe of approximately 10 min into a few hours. This is because PPV has a noncirculative, nonpersistent transmission pattern whereby

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The nucleotide sequence data for the mitochondrial cytochrome c oxidase I gene from *Myzus umecola* reported in this study appear in DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: B738877.1 and AB738876.1

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the virus can be transmitted only while the tip of the stylets of the aphid is in contact with the virus (Ferrerres and Moreno 2009). It is generally believed that noncircular viruses can be carried by various types of aphid nonselectively and that any aphid can acquire the virus from the sap of an infected plant. However, the transmission efficiency of potyviruses depends on the affinity between the HC-Pro of the virus and the stylet of the aphid; hence establishing carrier specificity (Wang et al. 1998).

It would not be easy to detect plant viruses in its insect vectors compared with infected plant materials such as leaves and branches, because the insect body is so small and the amount of virus is very low. Nevertheless, a few feasible detecting techniques for PPV in the aphid vectors using polymerase chain reaction (PCR)-based assays have been previously described (Cambra et al. 2004; Olmos et al. 1999, 2005; Wetzel et al. 1992). The methods suitable for an epidemiological investigation to handle many insect vector samples are not developed well enough to sufficiently maintain the level of high sensitivity for PPV detection.

The primary purpose of this study was to identify aphid species that transmit PPV in Japan and obtain information to be used to develop management measures. First, we trapped alate aphids in areas infested with PPV on a continuous basis and identified different species based on nucleotide sequence data to determine dominant aphid species and their outbreak patterns. Second, we tested each of the identified samples for the virus to ascertain high-risk aphid species that may transmit PPV in the infected areas, developing a new detection method for PPV in the vector aphids based on the nested PCR technique. Third, we tested transmissibility of the aphid species that were suspected to be the major vectors in the areas.

Materials and Methods

Aphid collection. Surveys to investigate annual fluctuations in the number of aphids in local orchards were conducted from April to December 2011 and from April to December 2012 in (i) a *P. mume* orchard in an area with a high density of PPV (D strain) occurrence in Ome City, Tokyo (hereinafter referred to as high-density area [HDA]) and (ii) a *P. mume* orchard in an area of Tokyo where existence of trees infected with PPV has yet to be surveyed (nonsurveyed area [NSA]). One set of four yellow insect-trapping adhesive plates was prepared so that they are facing in four different directions at each locations at a height of approximately 70 cm to attract alate aphids. All trapped aphid individuals were counted. Yellow sticky plates were set up every week for a period of 2 days, and the collected aphids were stored at -80°C .

Insecticides were applied as conventional maintenance procedures of cultivation in HDA. Flonicamid wettable powder was sprayed on 14 May 2011 and dinotefuran granulated wettable powder on 30 May 2011, whereas acetamiprid wettable powder was sprayed on 14 to 16 April 2012, flonicamid wettable powder on 19 to 21 May 2012, and dinotefuran granulated wettable powder on 1 to 4 June 2012. In contrast, no insecticide was used in NSA.

Nucleic acid extraction. From each survey area, 50 aphids (maximum) were collected from the adhesive sheets, and each aphid was individually placed in a 2-ml bead-beating tube with two 0.2-cm zirconia beads. A 200- μl aliquot of TRIzol Reagent (Life Technologies, Carlsbad, CA) and 75 μl of chloroform were added and shredded in a multibead shaker (Yasui Kiki, Osaka, Japan). Two cycles were performed under the following conditions: oscillation frequency, 2,500 rpm; shredding time, 60 s; rest time, 10 s. Thereafter, the contents were centrifuged for 15 min at 15,000 rpm and 4°C . A 100- μl aliquot of the resulting aqueous layer was transferred to a new 1.5-ml microtube, and 75 μl of 2-propanol and 1 μl of glycogen were added. After being stirred gently, the solution was centrifuged for 15 min at 15,000 rpm and 4°C . The supernatant was decanted, and 300 μl of 70% ethanol was added to the pellet. The solution was lightly stirred and then centrifuged for 15 min at 15,000 rpm and 4°C . The supernatant was decanted and, after complete desiccation in a centrifugal drier, the extract was dissolved in 10 μl of sterile distilled water. Specimens prepared as above were subjected to the following two detection systems separately for aphids and the virus.

Amplification and purification of aphid DNA by PCR. Following the TaKaRa Ex Taq protocol (Takara Bio, Shiga, Japan), 0.5 μl of nucleic acid extract, 1.5 μl of dNTP, 2 μl of $10\times$ Ex Taq buffer, 0.1 μl of Ex Taq, 1 μl of each primer (5 $\mu\text{M}/\mu\text{l}$), and double-distilled water (DDW) at the end were added to prepare 20 μl of the PCR solution. The primer set used comprised LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') to amplify 658 bp of the mitochondrial cytochrome c oxidase I gene region, which enables identification of aphids down to the species level (Folmer et al. 1994). PCR was performed using the following program: 94°C for 5 min; 5 cycles of 94°C for 1 min, 45°C for 90 s, and 72°C for 90 s; 35 cycles of 94°C for 1 min, 50°C for 90 s, and 72°C for 1 min; and 72°C for 5 min (Hebert et al. 2003). After the reaction, 4 μl of the PCR product was diluted in 36 μl of DDW, and electrophoresis was performed using LabChip GX (Perkin-Elmer, Waltham, MA) to verify the presence of bands. DNA was purified by mixing 2 μl of 0.1 $\times$ ExoSAP-IT (Affymetrix, Santa Clara, CA) with 5 μl of the PCR solution, using a program setting of 37°C for 30 min followed by 80°C for 15 min.

Sequence reaction and nucleotide sequence analysis. An aliquot of the sequencing reaction solution was prepared to a final volume of 10 μl using a BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) from 0.5 μl of purified DNA, 1 μl of BigDye Terminator v1.1, 1.5 μl of 5 $\times$ sequencing buffer, 1 μl of each primer (LCO1490 and HCO2198, 0.8 $\mu\text{M}/\mu\text{l}$), and DDW at the end. The sequencing reaction was performed using a program setting of 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The reaction products were purified using a Dye Terminator Removal Kit (Thermo Scientific, Waltham, MA); they were completely dried in a centrifugal drier and then dissolved in 10 μl of Hi-Di formamide (Life Technologies). A sample plate was set in the tray of 3130 Genetic Analyzer (Life Technologies) and sequencing was performed. The nucleotide sequence analysis was performed with CodonCode Aligner (Codon Code Corporation, Centerville, MA), and species were identified by National Center for Biotechnology Information BLAST database searches. The criterion adopted for species identification was that 0.2% or less discrepancy in nucleotide sequences against a reference species recorded in the database was considered to be the same (Footitt et al. 2008).

Aphid sampling and nucleic acid extraction for PPV detection. Virus screening was conducted for 50 aphids collected from the adhesive sheets from each of the sites to identify the aphid species. The negative control area for virus screening was set in Tsukuba City, Ibaraki Prefecture, which is approximately 100 km away from the PPV outbreak zone in Tokyo. Five aphids per week (maximum) were collected from the yellow adhesive plates installed in this area and surveyed in the same manner.

Virus detection in one aphid using nested reverse-transcription PCR. The reverse-transcription (RT)-PCR detection system for PPV in the vector aphids was newly developed for this study. The methods previously published (Olmos et al. 1999, 2005) seemed to be not suitable for an epidemiological investigation to handle many insect vector samples sufficiently, even if those would maintain the level of high sensitivity for PPV detection. Primary RT-PCR was performed using a PrimeScript One Step RT-PCR Kit (ver. 2; Takara Bio) in a test solution consisting of 2 μl of nucleic acid extract, 5 μl of $2\times$ 1-step buffer, 0.4 μl of PrimeScript 1 step Enzyme Mix, and 0.4 μl of each 5 μM primer, to which RNase-free water was added up to 10 μl in total volume. New designed primers PPV nest P1 (5'-CAGCCTGAATTTACAT TGTCATGG-3') and PPV nest R1 (5'-CATCAACTTCCTCT CGTCTTCTC-3') were used to amplify 1,000 bp of the Nib gene region, allowing the amplification of all D strains of PPV stored as nucleotide sequence databases in DDBJ, EMBL, and GenBank. PCR was performed using a program setting of 50°C for 30 min; 94°C for 2 min; and 35 cycles of 94°C for 20 s, 60°C for 10 s, and 72°C for 1 min. cDNA obtained was diluted 1:10 and subjected to secondary PCR.

Nested PCR was performed with KOD dash (TOYOBO, Osaka, Japan) after mixing 1 µl of cDNA that had been diluted by a factor of 10 with DDW, 1 µl of 10× buffer, 1 µl of 10 mM dNTPs, 0.1 µl of KOD Dash, and 0.2 µl of each 5 µM primer, to which DDW was added to make up 10 µl of test solution. The primer set used comprised the newly designed PPV P4 (5'-TGCAGTTCTCAA TATTCGTCTGGC-3') and PPV nest R2 (5'-TCCAAGTTGG GAAAA-3'), producing 346 bp of the cDNA fragment from the NIb gene region amplified in the first round. PCR was performed under the following conditions: 94°C for 2 min; 35 cycles of 94°C for 20 s, 60°C for 10 s, and 72°C for 20 s; and 4°C to stop the reactions. Finally, 4 µl of the resulting PCR product was diluted 1:10, and electrophoresis was performed using LabChip GX (Perkin-Elmer) to verify the presence of bands.

Statistical analysis. Using month, year, and the survey areas as explanatory variables, we applied logistic regression analysis according to the formula presented below to analyze the relationships between the explanatory variables and the incidence of PPV-positive insects, which was estimated from the number of PPV-positive insects surveyed in the study fields, as well as the reliability of that correlation.

$$\ln\left(\frac{p}{1-p}\right) = k + a \cdot \text{month} + b \cdot \text{area} + c \cdot \text{year} + d \cdot \text{month} \cdot \text{area} + e \cdot \text{month} \cdot \text{year} + f \cdot \text{area} \cdot \text{year} + g \cdot \text{month} \cdot \text{area} \cdot \text{year} + \varepsilon$$

Although sampling was conducted weekly in this survey, weekly changes were unsuitable as the explanatory variables for statistical processing due to the considerably low stability of the model; therefore, weekly data were combined to afford an explanatory variable on a monthly basis. Posthoc tests were performed using a G-test with Bonferroni-Holm correction to compare monthly variability. The statistical analysis was performed using JMP (ver. 5.1.2; SAS Institute Inc., Cary, NC).

PPV transmissibility evaluation. Seven different species of aphids (*Aphis craccivora*, *A. gossypii*, *A. spiraeicola*, *Melanaphis sacchari*, *Rhopalosiphum maidis*, *Myzus persicae*, and *M. persicae*) were captured live in Tsukuba City, Ibaraki Prefecture and maintained in a greenhouse at 27°C on host plant seedlings appropriate to each aphid species. Specimens were prepared to identify species based on conventional exterior morphology.

Live aphids maintained in the greenhouse were transferred to a plastic container using a thin paint brush and fasted for 1 h. Ten fasted apteran aphids were next placed on a *P. mume* leaf infected with PPV, and they were allowed to access acquisition feeding for 1 min after the aphids left on the leaf didn't move any more. The aphids were then transferred to a healthy *P. mume* seedling about 10 cm in height and allowed to access inoculation feeding overnight. The next day, the aphids were eradicated using an insecticide, and the inoculated *P. mume* plants were checked after 1 month to determine whether or not transmission had occurred based on the presence or absence of disease symptoms and the results of a serum test using Plum pox immune-chromato (Nippon Gene, Tokyo).

Results

Aphid outbreak trends in the surveyed areas. The number of aphids trapped on the yellow adhesive plates during the survey period in 2011 was 1,165 in HDA and 2,687 in NSA. The seasonal outbreak trends as illustrated in line graphs revealed that the number peaked in both areas during late April to late May, early June to late June, and late July to early September (Fig. 1). Another peak occurred in late November in NSA (Fig. 1b). The number of aphids trapped in 2012 was substantially lower than that in 2011, with 631 trapped in HDA (54.2% year-on-year) and 950 trapped in NSA (35.4% year-on-year) (Fig. 2). Due to the smaller sample size, no clear trapping peak was seen from the data calculated based on weekly collections. However, seasonal variation trends in the number of trapped aphids were the same as those observed in the previous year, with many outbreaks evident during parts of the spring, summer, and fall.

Dominant aphid species in the survey areas. Nucleotide sequence analysis was performed on 3,267 aphids trapped on the yellow adhesive plates during the survey period. In all, 45 aphid species were identified in HDA, 48 species in NSA, and 64 species in total from the two sites combined. Of those species identified, 13 species were known to transmit PPV (data not shown). The species accounting for ≥2% of the total number of samples for which sequence analysis was performed were selected as dominant species (Wallis et al. 2005). Outbreak trends for these dominant species by survey year and survey area are described below.

Year 2011. In total, 888 aphids were identified in HDA. Their seasonal trends revealed that *A. craccivora*, *A. gossypii*, *A. spiraeicola*, and *Tinocallis zerkowae* were the four dominant species during May and June, when the number of aphids trapped on the adhesive boards increased. *A. gossypii*, *A. spiraeicola*, *Melanaphis sacchari*, and *R. maidis* were the four dominant species trapped in August, when the second highest number of aphids was trapped. *Phorodon humuli* was prominent beginning in November and *A. spiraeicola* was trapped throughout the year (Fig. 1a).

In total, 982 aphids were identified in NSA. Major fluctuations were observed in the number of aphids trapped on the adhesive boards and there was a clear segregation between different species according to trapping peaks. Whereas *A. gossypii* and *A. spiraeicola* were dominant in late April and May, they were clearly replaced by *A. craccivora* and *T. zerkowae* in June. In addition to these four species that occurred in the spring, *M. sacchari* and *Shivaphis celti* were also dominant in late July and August. A diverse range of aphids occurred in November, including *A. gossypii* and *A. spiraeicola* (Fig. 1b).

Year 2012. In total, 588 aphids were identified in HDA. When compared with the figures from 2011, no clear peaks were observed in 2012 in the numbers of aphids trapped, with minor peaks in June, September through early October, and November. *A. craccivora*, *A. gossypii*, *A. spiraeicola*, and *Hyalopterous pruni* were the four dominant species in June, whereas *A. spiraeicola* was almost exclusively found from September to early October. *A. gossypii*, *P. humuli*, *R. padi*, and *R. rufiabdominalis* were dominant in November. *A. spiraeicola* was trapped throughout the season in 2012, similar to the trend in 2011 (Fig. 2a).

In total, 809 aphids were identified in NSA. No clear peaks were observed in the number of aphids trapped, similar to the pattern observed in HDA in the same year. After sporadic occurrences in mid-June, aphids continued to occur at a moderate level between early August and early December. *A. spiraeicola* was dominant in mid-June, whereas *A. gossypii*, *Lipaphis pseudobrassicae*, and *S. celti* were the three dominant species from early August through September. These species were replaced by *A. spiraeicola* and *P. humuli* from October to early December (Fig. 2b).

When the results of the surveys from both years were taken as a whole, it was clear that there was a major difference in the occurrence patterns for dominant aphid species in the surveyed areas. Despite there was being at least seven dominant species in HDA in 2011 (Fig. 1a), *A. spiraeicola* accounted for the vast majority in 2012 (Fig. 2a). Similarly, in NSA, various aphid species occurred at trapping peaks in 2011 (Fig. 1b) but a limited number of species, such as *A. spiraeicola* and *S. celti*, were found in 2012 (Fig. 2b). On the other hand, similar trends were observed between HDA and NSA with regard to the seasonal trends in dominant aphid species during each year of the survey. In 2011, *A. craccivora*, *A. gossypii*, *A. spiraeicola*, and *T. zerkowae* were common in both areas as dominant species in the spring, whereas *A. gossypii*, *A. spiraeicola*, and *M. sacchari* were common in the summer; *A. spiraeicola* was continuously dominant beginning September in both areas (Fig. 1). Likewise, in 2012, *P. humuli* was dominant both in HDA and NSA in November and later in the year, and *A. spiraeicola* was trapped throughout the year in both locations, although the dominant species were different between the two areas only between early August and September (Fig. 2).

Based on nucleotide sequence analysis, 12 aphid species were identified as dominant in this study (Table 1). Of these, the following five species accounted for ≥2% of the total in both HDA and NSA in both 2011 and 2012: *A. spiraeicola*, *A. gossypii*, *A. craccivora*,

R. rufiabdominalis, and *H. pruni*. The other seven species accounted for <2% in either one of the survey areas or one of the survey years. Among the dominant species, *A. spiraeicola* and *A. gossypii* showed the least change year-to-year and showed a dominance ratio of 10% or more in both areas and in both years. The species trapped most frequently during the survey period was *A. spiraeicola*, which accounted for approximately 26% (845 aphids) of the total.

Virus-positive aphid rate in the survey areas. The 3,276 samples for which nucleotide sequence analysis was performed were tested for the presence of PPV. Of all the samples, 126 tested positive (55 individuals in HAD and 71 in NSA), sharing 25% in HAD and 31% in NSA of *A. spiraeicola* as highest infectious species among the positive aphids, followed by *T. zelkowae* in HAD and *A. gossypii* in NSA (Fig. 3). In contrast, all 352 samples from Tsukuba City, which was set up as a negative control area, tested negative for the virus during the survey period. For viruses such as PPV transmitted in a nonpersistent manner, the risk of disease spread increases with the number of flying alate aphids that are possibly able to transmit the virus in an area including contaminated orchards. From the perspective of formulating prevention and eradication measures, it is important to investigate the periods and locations in which the risk of PPV transmission by aphids is high.

In this study, statistical analysis was conducted to discern factors that contributed to the increase in the number of virus-infected samples using “survey area”, “survey year”, and “survey month” as main effects. In addition to the aforementioned main effects, interactions produced between two or three of these factors were also examined. Furthermore, because data from the months of April to December in both the survey years were insufficient in the aphid sample size to form a population, which could compromise the reliability of the statistical tests, they were excluded from the analysis. Under these statistical conditions, the effects of survey month and tertiary interaction within a combination of survey area–survey year–survey month were significant when survey month was the only factor ($P < 0.0001$) and also when a tertiary interaction was taken into account ($P = 0.0359$); thus, significant differences were acknowledged in these two patterns (Table 2). A significant difference in the effect of survey month suggests that the appearance of virus-infected samples differed from month to month.

In our first attempt of statistical analysis, effects of interactions were rather difficult to interpret. Therefore, based on our judgment that it would be more straightforward to summarize data by year to explain interaction effects, we performed statistical analyses again for the 2011 and 2012 survey results separately. As a consequence,

Fig. 1. Outbreak trends for dominant aphid species in 2011 in the survey areas. **A**, Plum pox virus (PPV) high-density occurrence area (HDA). **B**, PPV nonsurveyed area (NSA). Bar graph, number of dominant aphids identified (left axis); line graph, number of aphids trapped on yellow adhesive boards (right axis). Minor species = aphid species accounting for <2% of the total number. Others = samples with no hits in a BLAST search and samples that could not be identified due to a $\geq 0.2\%$ difference from the nucleotide sequences in database.

the only significant difference of the effect of month was shown in 2011 (Table 3, $P = 0.0032$). No significant difference was detected for survey area or for the interaction of survey area–survey month. Similarly, for 2012, a significant difference of survey month only was observed, whereas the interaction effect between the location and the survey date was at an insignificant level (Table 4, $P = 0.0012$).

No significant difference was detected between the two survey areas for each year; therefore, we decided to treat the difference between the two areas as negligible. We summed the number of aphids that tested virus positive in both areas for each year and calculated the monthly virus-positive insect rate by considering only the survey month. The results showed that the virus-positive insect rate for May to September 2011 was not significantly different at a range of 0.9 to 2.0%, but it increased to 8.1% in October and then decreased to 3.4% in November. This means that the virus-positive insect rate is significantly higher in the early to late fall (when the total number of trapped aphids was lower) than that during the spring or summer period (when the number of trapped aphids was higher) (Fig. 4a). In 2012, no significant difference was seen in the virus-positive rate between May and August at 1.4 and 1.6%, respectively; on the other hand, it was significantly higher at over 5.2% in June, July, and

September through November. This was particularly the case in October, when the virus-positive rate was significantly high at 12.7% (Fig. 4b). A comparison of the calculated virus-positivity rates between 2011 and 2012 showed that, although results are different in terms of percentages, both years shared the same occurrence patterns for virus-positive insects. These survey results clearly indicate that virus-positive insect rates fluctuate mainly according to the season.

Evaluation of PPV transmissibility. We compared the transmissibility of PPV by 5 of the 12 dominant species of aphids that were trapped (*A. craccivora*, *A. gossypii*, *A. spiraeicola*, *M. sacchari*, and *R. maidis*), as well as *Myzus mumecola* and *M. persicae*, from infected to healthy *P. mume* (Table 5). The species with the highest transmission rate was *M. persicae* at 24.4%. *A. spiraeicola* had the second highest transmission rate (13.1%). Transmission rates for *A. craccivora* and *A. gossypii* were relatively low (5.3 and 3.7%, respectively). Transmissibility was not detected for *Melanaphis sacchari*. It is noteworthy that PPV transmissibility was shown for the first time for *Myzus mumecola* and *R. maidis* in this study. Infection transmitted by *M. mumecola* was identified in 5 of 41 plants (12.2%), showing a certain level of transmissibility. Infection by *R. maidis* was also found in 1 of 29 plants (3.4%).

Fig. 2. Outbreak trends for dominant aphid species in 2012 in the survey areas. **A**, Plum pox virus (PPV) high-density occurrence area (HDA). **B**, PPV nonsurveyed area (NSA). Bar graph, number of dominant aphids identified (left axis); line graph, number of aphids trapped on yellow adhesive boards (right axis). Minor species = aphid species accounting for <2% of the total number. Others = samples with no hits in a BLAST search and samples that could not be identified due to a $\geq 0.2\%$ difference from the nucleotide sequences in database.

Discussion

In terms of the 2011 aphid occurrence fluctuation pattern, trapping peaks were observed in the spring, summer, and parts of the fall. However, based on the results of virus detection tests and statistical analyses, the period with the highest virus-positive insect rate was the fall, including October at 8.1% and November at 3.4%, which was not understood for some reason. The average temperature during this period of the year rose to about 31°C in the summer from 12°C in the spring, and descended to about 17°C from summer to autumn, according to an official record of the Japan Meteorological Agency. These results suggest that, rather than spring and summer, when the number of trapped aphids is relatively high, fall is the period with the greatest risk of PPV outbreaks, when the rate of virus-positive insects is increased. In fall 2011, *A. gossypii*, *A. spiraeicola*, and *P. humuli* were shown to be dominant species. At that time, the species that were found with high numbers of virus-positive insects were *A. fukii*, *A. gossypii*, *A. spiraeicola*, and *T. zelkowae*. Two species with major occurrence in the fall, *A. gossypii* and *A. spiraeicola*, were among those virus-positive insects.

Although the 2012 aphid occurrence pattern was unclear, trapping peaks were observed in the spring, summer, and fall. The change of average temperature this year was about 12°C in the spring to 33°C in the summer and descended to about 15°C in autumn, the same as above. Based on the results of virus detection tests and statistical analysis, in addition to the period of September through November, when the virus-positive insect rate ranged from 6.1 to 12.7%, periods other than the fall season also demonstrated relatively high virus-positive rates, including the months of June at 6.6% and July at 5.2%. Therefore, the PPV transmission risk was likely to be high among aphids occurring during June, July, and fall 2012. In June and July, *A. spiraeicola* and *H. pruni* were dominant whereas, in the fall, *A. gossypii*, *A. spiraeicola*, and *P. humuli* were dominant. Species that were actually high in the number of virus-positive insects were *A. gossypii*, *A. spiraeicola*, *P. humuli*, and *H. pruni*; thus, the composition of aphid species occurring in the fall exactly matched that of species with a high number of virus-positive insects. This was particularly the case with *A. gossypii* and *A. spiraeicola*, which both occurred during the fall for two consecutive years.

PPV infections in Japan are similar to epidemiology in the Mediterranean Sea area. It has previously been described that the proportions of aphid species captured in the *Prunus salicina* orchards in the Valencia region, Spain in 3 years (1993, 2002, and 2003) were *A. spiraeicola* (43%), *A. gossypii* (18%), *H. pruni* (6%), *Brachycaudus prunicola* (6%), *A. craccivora* (3%), *M. persicae* (2%), and other species (22%); and the percentage of virus-positive aphids was 11.9% in May (Cambra et al. 2004). In the Antakya-Hatay region of Turkey, the dominant aphid species were *A. spiraeicola* and *A. gossypii*, showing a peak in May in both 2011 and 2012; and the percentages of PPV-viruliferous aphids were 12.5% in *A. spiraeicola*, 12.42% in *A. gossypii*, and 11.11% in *H. pruni* (Kaya et al. 2014). Based on the fact that the virus-positive insect rate for the both *A. spiraeicola* and *A. gossypii* was high, they are likely to pose a high PPV transmission risk. In previous studies aimed at detecting PPV in aphids, it is known that the virus could be detected in nonvector species (Olmos et al. 1999, 2005). Therefore, aphids may carry the virus in their intestinal tract after having fed on a PPV-infected tree without being a vector of PPV. However, the detection of PPV in flying aphids is a very important indication that active sources of PPV are present in or around the area, meaning a high risk of spreading PPV disease in cases where PPV-positive aphids, particularly *A. spiraeicola* and *A. gossypii*, were detected in the area.

Although *M. persicae* demonstrated a relatively high PPV transmission rate of 22.4% in experimental transmission tests, a rate of 63% has been reported in cases of peach-to-peach transmission using the same PPV-D strain (Gildow et al. 2004). The transmission efficiency observed with *M. persicae* in this study was lower compared with the results from overseas. The transmission rate for *A. spiraeicola* was 13.1% and it was the second highest after *M. persicae* in our study. On the other hand, *A. gossypii* has been reported as an

inefficient vector based on results from experiments conducted in North America and Europe (Labonne et al. 1995), and this study also showed a low rate of 3.7%. Meanwhile, the PPV transmissibility was newly confirmed for *M. mumeicola* and *R. maidis*. *M. mumeicola* is known to be parasitic on *P. mume* and occurs in large numbers during the period of foliation in March through April. In this study, only 14 individuals of this species were trapped and none tested positive for PPV. However, because the species showed the second highest rate of transmission efficiency after *A. spiraeicola* at 12.2% in the transmission test, we recognize the need for more detailed surveys of its behavior in early spring and the occurrence patterns of its alate form. Although *R. maidis* was found to be a dominant species in this study, it is believed to be an inefficient vector because it had a relatively low transmission rate of 3.4% in the tests. In the case of *Melanaphis sacchari*, the presence of virus-positive aphids was verified but it did not transmit PPV experimentally. The vector specificity of *Potyvirus* spp., including PPV, has been known, likely because viral particles are unable to attach to the tip of stylets of certain aphid species, which is a required process for transmission (Uzest et al. 2007). It was evident that *M. sacchari* was sucking sap from PPV-infected plants but its PPV transmissibility was not verified; thus, it was considered not to be an insect vector at this point.

It was suspected that *A. gossypii* contributed to the PPV transmission in the affected areas based on the results of the 2-year field survey and PPV transmission tests showing that the species occurred in large numbers during the fall, a large number of virus-positive insects were detected, and it was proven experimentally that it transmitted PPV. However, because its PPV transmission efficiency was relatively low, it was surmised that it was not the main vector species. In contrast, *A. spiraeicola* occurred in large numbers in the fall, during which the PPV transmission risk was high, and it also showed a relatively high transmission efficiency of 13.1% in PPV transmission tests. These results suggest that *A. spiraeicola* could be among main aphid species contributing to PPV transmission in HDA in Tokyo. The virus detection tests revealed many virus-positive insects among *A. fukii*, *T. zelkowae*, *H. pruni*, and *Phorodon humuli*. However, because nested RT-PCR is an extremely sensitive method, it is important to note that the PPV transmission may not necessarily follow just because virus-positive results are obtained. Because PPV is a nonpersistent, noncirculating type of virus, it loses its capability to be passed on within 1 h of being acquired by an aphid. In contrast, it is possible to detect PPV from artificially created viruliferous insects 24 h after they become carriers. In other words, aphids that have lost the ability

Table 1. Dominant aphid species in two survey areas during a 2-year survey period^a

Species	HDA		NSA		Total of both areas	
	2011	2012	2011	2012	N	Percent
<i>Aphis spiraeicola</i> ^b	201	226	190	228	845	26
<i>A. gossypii</i> ^b	129	78	118	81	406	12
<i>A. craccivora</i> ^b	92	19	106	19	236	7
<i>Tinocallis zelkowae</i>	50	14	148	9	221	7
<i>Shivaphis celti</i>	14	1	37	130	182	5
<i>Phorodon humuli</i> ^b	29	30	13	66	138	4
<i>Melanaphis sacchari</i>	50	6	59	7	122	4
<i>Rhopalosiphum ruftabdominalis</i>	25	20	24	31	100	3
<i>R. maidis</i> ^b	41	29	21	0	91	3
<i>Hyalopteris pruni</i> ^b	20	35	19	14	88	3
<i>R. padi</i> ^b	10	22	17	18	67	2
<i>Lipaphis pseudobrassicae</i>	8	3	16	39	66	2
Minor species ^c	97	52	97	72	318	10
Others ^d	122	53	117	95	387	12
Total	888	588	982	809	3267	100

^a HDA = high-density area and NSA = nonsurveyed area.

^b Species of aphids reported as *Plum pox virus* vectors.

^c Species accounting for <2% of the total number of aphids captured in both areas.

^d Species with no hits in a BLAST search.

to transmit PPV as well as aphids that have acquired the virus but are not capable of transmitting it, such as *M. sacchari*, can be detected as virus-positive insects with nested RT-PCR, and not all virus-positive insects are necessarily confirmed as PPV carriers. Thus, it is essential to perform separate PPV transmission tests to verify the capability and efficiency of transmission for these four aphid species.

Furthermore, almost no *Myzus persicae* aphids, the main PPV transmitter overseas, were trapped in this study. This result suggests that there is difference among aphid species in terms of the level of their attraction to yellow adhesive plates. When yellow versus green water traps are set to trap *A. spiraecola*, aphids are known to be attracted to the yellow traps in three times higher numbers compared with those attracted to the green traps (Avinent et al. 1991). Although *A. spiraecola* was attracted in the highest numbers in our present study, it is conceivable that each aphid species shows a varied level of affinity toward the yellow adhesive plates, and new studies using other survey methods, such as water traps, are needed. Actually, a certain number of *M. persicae* individuals were captured in a water trap method carried out at the same area above as a preliminary study (data not shown).

Because PPV is noncirculative and nonpersistent, transmission from an infected tree to a healthy tree must occur during a short time period. Therefore, the dominant vector changes depending on the types of aphid that are prevalent in a particular area. Three major vectors known in Eastern Europe are *B. helichysi*, *H. pruni*, and *P. humuli* whereas major vectors in Western Europe and North

Table 2. Results of a logistic regression analysis for the factors of survey year, survey area, survey month, and interactions among the three variables

Factor	df	G	P
Survey year	1	5.38302e-6	0.9981
Survey area	1	4.84864e-6	0.9982
Survey year × survey area	1	1.69527e-6	0.9990
Survey month	8	42.5094209	<0.0001 ^a
Survey year × survey month	8	4.40648246	0.8187
Survey area × survey month	8	8.63135991	0.3743
Survey year × survey area × survey month	8	16.4849223	0.0359 ^a

^a Significant difference.

Fig. 3. Proportion of 126 alate aphid species showing *Plum pox virus* (PPV) positive reaction in a 2-year survey. The outside of the duplicate pie graph represents the high-density occurrence area (HDA) and the inside represents the PPV nonsurveyed area (NSA).

America are *A. spiraeicola* and *M. persicae* (Gildow et al. 2004). Transmission efficiency differs according to the biotype even among the same aphid species (Lupoli et al. 1992). Although *H. pruni* is a major vector in Eastern Europe, in contrast, a case of the species transmitting the virus has not yet been reported in Western Europe (Gildow et al. 2004). It is important to clarify the outbreak patterns of these major transmission vectors to prevent and eradicate infection. An investigation conducted in a peach orchard in Pennsylvania over a 2-year period revealed that outbreak peaks for *A. spiraeicola* and *M. persicae* were in June to July and September to October, respectively (Wallis et al. 2005). An investigation conducted in a plum orchard over a 2-year period in Valencia, Spain, revealed that *A. spiraeicola* and *A. gossypii* accounted for 80% of the 6,000 aphids characterized and that the outbreak peak was in May (Capote et al. 2008). These data illustrate that dominant aphid species and outbreak peaks vary in different geographic regions. In Japan, where the invasion of PPV was only recently confirmed, the aphid species that act as major vectors have yet to be identified.

The increased numbers of virus-positive insects in the fall are presumably attributable to an increased proportion of alate aphids sucking sap from green branches of *Prunus mume*, newly developed in summer, during the same period due to the presence of some aphid species that switch their hosts and return to *P. mume* or some other species that move away from *P. mume* to a secondary host before winter hibernation (Aoki 2000). In fact, some green branches of PPV-infected *P. mume* in the fall were tested positive for the virus (K. Maejima, personal communication). In addition, developing leaves in this period would be better for alate aphids that suck sap because the softness of those leaves is similar to that of leaves developed in the last spring, just before the summer. In contrast, a peak of the PPV-viruliferous aphids in the Mediterranean Sea area has been previously indicated to be in May (Cambra et al. 2004; Kaya et al. 2014). The cause of this peak in the Mediterranean Sea area may be that the alate aphids prefer the young, soft, developing leaves and shoots of *P. salicina* during early spring to those developing in late spring, just before the summer. In Japan, however, the aphids may prefer the green branches that characteristically grow in summer for *P. mume*, leading to the increased numbers of virus-positive insects in the fall. It is unclear what kinds of natural phenomenon cause such a big difference in whether a peak appears in late spring or fall; viruliferous aphids have occurred in both Japan and the Mediterranean Sea area thus far. In our experiments of aphid transmission, only aphids allowed to acquire PPV on young and soft leaves showing symptoms of PPV-infected *P. mume* could transmit the virus, whereas aphids failed to transmit PPV when old, hard infected leaves were used, even those showing indications of typical symptoms of the disease (data not shown). It is important to conduct further surveys in the field and clarify the ecological mechanism of PPV infection of plants because virus-positive insects are detected even after

infected trees are cut down, and species of trees that are infected other than *P. mume* may also play a part in the mechanism of virus transmission, especially for the increase of PPV-positive aphids in the fall.

Fig. 4. Virus-positive rate of aphid species in the survey areas from each survey year: **A**, 2011 and **B**, 2012. Bar graph, virus-positive aphid ratio (%; left axis); line graph, number of aphids trapped on yellow adhesive boards (right axis). Virus-positive rates for individual species were calculated based on the following equation.

$$p1(n) = P(n) \times \frac{x1(n)}{X(n)}$$

Total viruliferous insect rate, $P(n)$; viruliferous insect rate of species, $p1(n)$; total number of trapped individuals, $X(n)$; number of trapped individuals by species, $x1(n)$. Different letters indicate significant differences ($P < 0.0001$) between treatments after a G-test for contingency table (Bonferroni-Holm's corrected) followed by a Posthoc test. Bars on bar graphs represent standard errors.

Table 3. Results of a logistic regression analysis for the 2011 data, with factors of survey area, survey month, and interaction between the two variables

Factor	df	G	P
Survey area	1	5.10488e-8	0.9998
Survey month	6	19.6150276	0.0032 ^a
Survey area × survey month	6	8.79110311	0.1857

^a Significant difference.

Table 4. Results of a logistic regression analysis for the 2012 data, with factors of survey area, survey month, and interaction between the two variables

Factor	df	G	P
Survey area	1	1.01103607	0.3147
Survey month	6	22.0686753	0.0012 ^a
Survey area × survey month	6	12.4479724	0.0527

^a Significant difference.

Table 5. Plum pox virus (PPV) transmission by seven aphid species in laboratory assays

Species	Inoculated	Infected	Transmission (%)
<i>Myzus persicae</i> ^a	45	11	24.4%
<i>Aphis spiraeicola</i> ^a	61	8	13.1%
<i>M. mumeicola</i>	41	5	12.2%
<i>A. craccivora</i> ^a	19	1	5.3%
<i>A. gossypii</i> ^a	27	1	3.7%
<i>Rhopalosiphum maidis</i>	29	1	3.4%
<i>Melanaphis sacchari</i>	15	0	0.0%

^a Species of aphids reported as PPV vectors.

Although it is required to cut down infected trees to eradicate PPV in Japan, it is also important to decrease the population of the vector aphids that transmit PPV to prevent a further spread of the infection. In this study, application of insecticide three times during the spring in a customary manner made it possible to reduce the aphid numbers in HDA by approximately 50% compared with the level in NSA. Because the aphid occurrence peaks in early May, mid-June, and late August, insecticide should be sprayed 2 weeks prior to each peak for its effectiveness. Furthermore, based on the present virus test results, spraying insecticide in conjunction with the October and November peaks, when a high number of PPV-positive aphids were seen, is also desirable. Although insecticide treatments in the orchard may prevent the spreading of PPV, they may have very little effect on aphids passing through crops, which are the most efficient vectors of nonpersistent viruses such as PPV because they are not colonizers of the hosts of interest. In order to control PPV, it is necessary to carry out measures to reduce the population of alate aphids throughout the area.

Because Japan has a short history of dealing with PPV, many aspects of the disease remain to be elucidated. Therefore, it is recommended that surveys, including virus testing, be continued in local fields where PPV has occurred. In addition, in order to evaluate the risk of PPV spread more accurately, the transmissibility of aphid species suspected of being PPV carriers in local fields should be assessed and the risk analysis of infection should be conducted for trees in these areas. Through these studies, the spread of this viral infection is preventable by accumulating epidemiological knowledge, such as the ecological mechanism of PPV vector insects, and by identifying an optimum period for decrease in the numbers of aphid populations. We hope that our steady ongoing efforts will contribute to effective measures for the future eradication of PPV.

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