Mutual complementarity among diverse pollinators as a mechanism underlying open insect pollination in Japanese pear orchards

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Abstract
Cultivated Japanese pear varieties, mostly showing self-incompatibility, require pollen transfer from other varieties. To elucidate the underlying mechanisms of open Japanese pear insect pollination, we first collected flower-visiting insects using plastic vials and sticky traps in orchards located at different regions in Japan. Results showed that insects assigned to the families Andrenidae (Hymenoptera), Apidae (Hymenoptera), Halictidae (Hymenoptera), Syrphidae (Diptera) and Empididae (Diptera) are abundant in the orchards. Second, we restricted the flower-visiting insects to access Japanese pear flowers using bags with mesh sizes of 0, 2 and 3.5 mm. Results indicated that insects which allowed to pass through bags with 3.5-mm mesh size but not through bags with 2-mm mesh size contribute primarily to pollination, represented as the fruit-set ratio and seed number. Third, we measured head and thorax widths of the flower-visiting insects and counted pollen grains on their body surfaces to estimate their pollination potential. Results indicated that insects assigned to the families Andrenidae, Halictidae, Syrphidae, Bibionidae (Diptera) and Muscidae (Diptera), including species with both widths smaller than 3.5 mm, harbour large quantities of Pyrus pollen grains, in addition to Apis mellifera (Apidae) with both widths greater than 3.5 mm. Consequently, the families Andrenidae, Apidae, Halictidae and Syrphidae might be the most important insect families for Japanese pear pollination. However, species identification of the flower-visiting insects showed no common key species that contribute remarkably to pollination services other than A. mellifera. Consecutive insect collection using plastic vials and sticky traps demonstrated that compositions of the flower-visiting insects are fluctuating continuously in the orchards. Nevertheless, year-to-year fluctuation of the fruit-set ratio was less pronounced in open-pollinated orchards than in hand-pollinated orchards. These results suggest that mutual complementation among diverse pollinator species might be the mechanism underlying open Japanese pear insect pollination.

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1 | INTRODUCTION

The importance of ecosystem services provided by pollinator insects for worldwide agricultural production, including that of fruit, has been well documented (Klein et al., 2003). For fruit production in Japan, the pollination services are estimated to be worth more than 200 billion JPY (ca. 1.8 billion USD) annually (Konuma & Okubo, 2015). This amount corresponds to ca. 30% of the total fruit output per annum. Japanese pear, Pyrus pyrifolia (Burm. f.) Nakai, is an important commercial fruit in Japan. Japanese pears originating from China have been cultivated for more than 1300 years (Saito, 2018). Commercial pear orchards in Japan were established in the late Edo era (1603–1887). Since then, over 1,000 cultivars have been developed (Saito, 2018). Currently, Japanese pears are produced throughout Japan, mostly for raw consumption. They are not cultivated in the Nansei islands (southwestern islands off Kyushu and in the Okinawa archipelago; Saito, 2018). In 2020, their cultivated area and yield in Japan were, respectively, 10,700 ha and 170,500 tons (https://www.maff.go.jp/).

Most cultivated Japanese pear varieties show self-incompatibility (Kikuchi, 1929; Kobayashi, 1971). Therefore, mixed planting with pollenizers has been practiced for pollen transfer among varieties. In the 1950s, hand pollination became common because of unidentified pollinator decline (The Japanese Society for Horticultural Science, 1973). However, hand pollination is remarkably costly and labour intensive. The short flowering period of Japanese pear and the changeable weather conditions, such as wind and rainfall, in early spring also might adversely affect hand pollination more than open pollination. Additionally, labour shortages exacerbated by Japan’s ageing economy has made hand pollination more unsustainable. For those reasons, open pollination by insect pollinators has attracted attention again for Japanese pear production.

Some insects, such as European honeybee Apis mellifera Linnaeus and Bombus spp. (Hymenoptera: Apidae), have been used industrially as pollinators for widely various agricultural crops, including Japanese pear. However, recent worldwide shortages of A. mellifera (Bosch & Kemp, 2002; Cane, 2005) and registration of Bombus terrestris (L.) as invasive alien species in Japan (Yoneda et al., 2008) have prompted the utilization of other pollinators. Osmia spp. (Hymenoptera: Megachilidae) have been used globally as pollinators in some fruit tree crops: for example, Osmia cornifrons Radoszkowski (Maeta & Kitamura, 1965; Sekita, 1998) for apple in Japan, O. lignaria (Say) for almond, apple, sweet cherry and apricot in North America (Bosch & Kemp, 1999; Torchio, 1985) and O. cornuta (Lateille) for almond, apple and pear in Europe (Bosch, 1994; Maccagnani et al., 2007; Vicens & Bosch, 2000). Reportedly, in open pollination of oriental persimmon, Bombus ardens Smith, 1879, distributed throughout almost all regions in Japan, functions as the most important pollinator species (key species) (Nikkeshi et al., 2019).

The importance of conditions of pollinator diversity, rather than having one or two key species, for pollination services has also been described (Blitzer et al., 2016). Reportedly, a diverse range of insects with functional diversity improved pollination services in apple orchards (Blitzer et al., 2016; Martins et al., 2015). Surveys of pollinator insects were conducted at some Japanese pear orchards during 1971–1973 (Kumakura et al., 1973; Okabe, 1973). However, little information is available about the presence or absence of key pollinator species and pollinator diversity, both of which can affect pollination services and subsequent fruit production of Japanese pears.

For this study, to elucidate the underlying mechanisms of open Japanese pear insect pollination, we first surveyed flower-visiting insects at Japanese pear orchards located in different regions in Japan. Second, we evaluated the flower-visiting insects for their contribution to pollination, represented as the fruit-set ratio and number of seeds, using bags with different mesh sizes. Third, we measured the head and thorax widths of the flower-visiting insects and counted the pollen grains on their body surfaces. Based on the results and data obtained from consecutive surveys of insects and fruit-set ratios, we discuss mechanisms underlying open insect pollination in Japanese pear orchards.

2 | MATERIALS AND METHODS

2.1 | Study sites

This study was conducted at five study sites in Japanese pear orchards of Tochigi (eastern Japan), Tottori (western Japan), central Kumamoto and southern Kumamoto (southern Japan). The survey in Tochigi was administered at the farm of Utsunomiya University (N36°48′E139°98′). The study site (3,600 m²) at the farm had a total of 71 trees of 10 varieties in 2018–2019. In 2020, 55 trees of nine varieties remained after old trees had been cut down. The study site, adjoining crop fields, semi-natural grasslands and orchards mainly cultivating grape, apple and chestnuts was surrounded by secondary Japanese cypress (Chamaecyparis obtusa) forest and rice paddy fields.

The surveys in Tottori were administered at two experimental orchards (Site 02 and Site 05; N35°28′E133°44′), Site 02 (5,000 m²) and Site 05 (1,800 m²) in the Horticultural Research Center, respectively, had 136 trees of seven varieties and 20 rows of seven varieties using the joint-tree training system (Shibata et al., 2011). The Horticultural Research Center, cultivating mainly Japanese pear and additionally cultivating oriental persimmon, citrus and apple, was surrounded by secondary forest consisting of cedar (Cryptomeria japonica) and Japanese cypress and crop fields. Both study sites, each of which was enclosed by a windbreak consisting of Distylium racemosum, sweet
viburnum (Viburnum odoratissimum), cedar and Japanese cypress, were 110 m distant from each other.

The surveys in central Kumamoto and southern Kumamoto were administered, respectively, at an experimental orchard of the Fruit Tree Research Institute (N32°41′E130°40′; 1,200 m²) and at a commercial orchard (N32°13′E130°54′; 2,062.5 m²). At the study sites in central Kumamoto and southern Kumamoto, 43 trees of six varieties and 68 trees of six varieties were planted respectively. The Fruit Tree Research Institute, where Japanese pear, oriental persimmon, peach, plum, grape and citrus were cultivated, was surrounded by secondary Quercus acutissima forest and by waste land. The study site in southern Kumamoto, adjoining orchards cultivating Japanese pear, chestnut and peach, was surrounded by secondary forests consisting of cedar and Japanese cypress. The direct distance between both study sites was ca. 70 km.

Open pollination has been practiced at study sites in Tochigi and southern Kumamoto. Hand pollination has been practiced at other study sites in Tottori and central Kumamoto. No managed pollinators have been introduced at the study sites. They were managed conventionally using insecticides, acaricides and fungicides. Wild ground-cover vegetation of the study sites was managed once or twice a month using mowing machines. The mowing height was around 40 mm in Tochigi and Tottori, 50–100 mm in central Kumamoto and 100–150 mm in southern Kumamoto.

### 2.2 Collection of flower-visiting insects in 2018 and 2019

Flower-visiting insects were collected directly on the flowers using 50-ml plastic vials in 2018 and 2019. Insect collection was done by three persons for 20 min every hour between 8:00 and 14:00 on April 5, April 6 and April 11 in 2018 and for a total of two hours by three persons between 9:00 and 10:00 and between 13:00 and 14:00 on April 16, April 19 and April 23 in 2019 at the study site in Tochigi. In Tottori, insects were collected for 30 min by two persons every two hours between 8:00 and 14:00 (Site 02) and between 8:30 and 14:30 (Site 05) on April 3 and April 8 in 2018 and for 30 min by one person every two hours between 8:00 and 14:00 (Site 02) and between 8:30 and 14:30 (Site 05) on April 12 and April 15 in 2019. In southern Kumamoto, insects were collected by one person without a set collection period on March 30 (9:37–15:56), April 3 (11:14–15:00), April 4 (10:06–13:59) and April 9 (10:25–13:54) in 2018 and for 15 min by two persons on April 4 (14:10–14:25), April 8 (11:10–11:25 and 14:30–14:45) and April 11 (11:00–11:15) in 2019. The collected insects were stored at −20°C until morphological identification to the family level.

### 2.3 Collection of insects using sticky traps

Results of the survey described above indicated that collection using plastic vials is unsuitable for trapping of some insect families, such as the family Anthomyiidae (data not shown). Consequently, we also surveyed insects inhabiting the study site in Tochigi using sticky traps. The trap comprised a white-coloured plastic board (140 × 340 mm) on which the top side is attached with a transparent sticky plastic sheet (No. 9850, Daiyko Giken-Kogyo Co., Ltd.). Using a trellis training system that is widely used for Japanese pear production in Japan (NARO, 2006), three traps were set horizontally to the ground from April 12 through April 26 in 2019 and from March 26 through April 9 in 2020. These traps were replaced with new traps on April 19 and April 2 in 2019 and 2020 respectively.

Collected insects were identified directly on the traps based on their morphological characteristics.

### 2.4 Species identification of insects collected in 2020

In 2020, flower-visiting insects were collected on April 2, April 15, April 16 and April 19 in Tochigi, on April 14, April 15 and April 17 in Tottori (Site 02) and on March 29, April 2 and April 6 in central Kumamoto to survey pollen grains on their body surfaces and to measure their head and thorax widths. The collected insects do not necessarily reflect the species composition of flower-visiting insects at the study sites. For example, A. mellifera was not collected in Tottori or central Kumamoto because sufficient samples for pollen grain counting were collected in Tochigi and possible contribution in Japanese pear pollination represented as fruit-set and seed numbers was shown in the study sites. For efficient pollen grain collection, 5-ml plastic vials were used in this survey. Collected flower-visiting insects were cooled immediately in a plastic box containing a refrigerant to prevent decomposition of the pollen loads on the hind legs and were stored at −20°C until pollen grain counting with subsequent morphological identification to the genus or species level.

Morphological characterization of the collected insects was conducted using microscopes (SZX10, SZX7 and SZ43; Olympus Corp.). The Hymenoptera and Diptera were identified morphologically, mainly based on reports by Tadauchi and Murao (2014), Nakayama (2009), Kano and Shinonaga (1968), Saigusa (2008) and Hardy and Takahashi (1960). Scientific names of the insects followed Tadauchi (2020) and Murao and Mita (2020) for Hymenoptera and Nakamura et al. (2014) for Diptera. After examination, the specimens were deposited in the Laboratory of Applied Entomology, School of Agriculture, Utsunomiya University (Diptera) and the Entomological Laboratory, Faculty of Agriculture, Kyushu University, Motooka, Japan (Hymenoptera).

Morphological identification of Delia platura, Episyrphus balteatus, Stomorhina discolor, Episyrphus balteatus, Eristalis tenax, Eupeodes corollae, Eristalis quinquelineatus, Eristalis cerealis, Helophilus eristaloides, Ferdinandeae cuprea and Calliphora nigrifrons was confirmed by molecular identification using cytochrome c oxidase subunit I sequences. DNA was extracted individually from collected insects using a sample preparation reagent (PrepMan Ultra; Applied Biosystems). Briefly, one of the hind legs introduced into 10 μl of the reagent was incubated at
95°C for 10 min and then at room temperature for 2 min. After centrifugation at 15,000×g for 2 min, the supernatant was recovered as a DNA sample. The supernatant (0.5 µl) was used for PCR amplification using primers, C1-J-1718 (5′- ggaggatttggaaattgattagttcc-3′) and C1-N-2776 (5′- ggataatcagaatatcgtcgagg-3′) (Simon et al., 1994). Quick Taq HS DyeMix (Toyobo Co. Ltd.) was used for PCR. The PCR conditions were one cycle of 3 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 45°C and 1 min at 72°C, with final extension of 72°C for 7 min. Nucleotide sequencing was conducted using C1-J-1718, a dye terminator cycle sequencing kit v3.1 (Applied Biosystems), and a DNA sequencer (3500 Genetic Analyzer; Applied Biosystems).

2.5 Pollen grain counting and head and thorax width measurements of insects collected in 2020

Estimations of the numbers of Pyrus pollen grains on the body surfaces of the flower-visiting insects collected in 2020 were conducted according to the method reported by Nikkeshi et al. (2019). Briefly, hind legs of collected insects, containing pollen loads that were less useful for pollination, were removed using scissors. After the body remains were returned into the vial filled with 0.4 M sucrose solution (300–700 µl), it was mixed gently by shaking. The pollen grains disengaged from the body surfaces were collected in the sucrose solution. The pollen grains in 10 µl of the sucrose solution were counted for the five replicates using a microscope (BX40; Olympus Corp.). Based on the average number of pollen grains, the total number of pollen grains on the body surface was calculated in accordance with the initial solution volume. The remaining insect samples were stored in 99.5% ethanol until morphological identification.

Head and thorax widths of the flower-visiting insects collected in 2020 were measured for estimation of body size effects of flower-visiting insects in the access restriction treatments (see below). Microscopes (SZX10, SZX7 and SZ43; Olympus Corp.) with eyepiece micrometres were used for measurements.
Contribution of flower-visiting insects with different body sizes to pollination were examined in Tottori and central Kumamoto in 2019 and in Tochigi in 2019 and 2020 using bags with different mesh sizes. This experiment used three access restriction treatments: inflorescences with buds before floral opening were covered with (1) a white-coloured polypropylene unwoven cloth bag (0M), (2) a white-coloured polypropylene bag with a 2-mm mesh (2M), or (3) a white-coloured polypropylene bag with a 3.5-mm mesh (3.5M) before flowering, as described by Nikkeshi et al. (2019). As controls, hand pollination followed by open pollination (HPO; only in Tottori and central Kumamoto) and open pollination (OP) were adopted for this experiment.

In Tochigi, surveys were conducted twice, using six trees (five Nikkori-variety and one Chojuro-variety trees) in 2019 and using six Chojuro-variety trees in 2020. Each tree contains five replicates of respective treatments. Consequently, each treatment was set to be 30 plots. However, some plots were lost to damage by crows in 2019. Other surveys were also conducted in 2019 using three Shinkansen-variety trees, each of which contains 15 replicates of respective treatments (a total of 45 plots for each treatment) in Tottori (Site 02 and Site 05) and using three Hosui-variety trees, each of which includes seven replicates of the respective treatments (a total of 21 plots for each treatment) in central Kumamoto. Fruits that had set and seeds that had set in the fruits of the respective treatments were counted.

The numbers of fruits in the OP treatment were examined in Tochigi and Tottori in 2021, in central Kumamoto in 2020 and in south Kumamoto in 2020 and 2021. In Tochigi, a survey was administered using three trees for six varieties: Akiakari, Akizuki, Hosui, Kanta, Kosui and Nikkori. Each tree contains five replicates to set 15 plots for the respective varieties. Surveys were conducted using four Hosui-variety trees, each of which contains 50 replicates (a total of 150 plots) in Tottori, using four Hosui-variety trees, each of which contains three replicates (a total of 12 plots) in central Kumamoto and using four Hosui-variety trees, each of which contains four trees for four varieties: Hosui, Kosui, Akizuki and Syurei. Each tree contains 6–8 replicates to set 24–26 plots for the respective varieties.

### 2.7 Statistical analysis

The fruit-set ratio and the number of seeds were compared among pollination treatments using a generalized linear mixed model (GLMM), as described by Nikkeshi et al. (2019). Briefly, the fruit set and the numbers of seeds, pollination treatment and tree ID were set, respectively, as response variables, an explanatory variable and a random effect. Tukey’s post hoc test was applied to pairs of pollination treatments for a significant difference detection. Statistical analyses described above were performed using software (R ver. 3.5.1; R development Core Team, 2018). The glht function in the multcomp package (Hothorn et al., 2008) and Wald’s test with the GLMM function in the glmmADMB package (Skaug et al., 2016) were adopted, respectively, for the Tukey’s test and significance assessment of the coefficients.
3 | RESULTS

3.1 | Collection of flower-visiting insects

Flower-visiting insects captured per person per hour in the three regions are presented in Table 1. In all, 297 hymenopterans were captured as flower-visiting insects in Tochigi in 2018. The most abundant hymenopteran family/species was *A. mellifera* (Apidae), followed by the families Tenthredinidae and Andrenidae. Among 174 flower-visiting dipterans, Syrphidae and Empididae were abundant families.

In all, 172 insects assigned to Hymenoptera and Diptera were captured in Tochigi in 2019. Andrenidae and Syrphidae were, respectively, the most abundant hymenopteran and dipteran families. However, *A. mellifera* and the family Empididae were less abundant in 2019. No Tenthredinidae was captured in 2019.

In Tottori, in all, 9 and 55 insects were, respectively, collected in 2018 and 2019. In 2019, insect families other than the family Syrphidae were less abundant.

In southern Kumamoto, in all, 164 and 98 hymenopterans were captured, respectively, in 2018 and 2019. The most abundant hymenoptera was the family Andrenidae, followed by Halictidae in both years. Among 220 dipterans collected in 2018, Empididae and Syrphidae were the most abundant families. In 2019, the families Syrphidae and Scathophagidae were the most abundant. The family Empididae was relatively less abundant.

| Table 1 | Flower-visiting insects captured in Tochigi, Tottori and southern Kumamoto in 2018 and 2019 |
|---|---|---|---|---|---|---|---|
| Order | Family | Hymenoptera |  |  |  |  |  |
|  |  | 2018 | 2019 | 2018 | 2019 | 2018 | 2019 |
| Hymenoptera | Andrenidae | 2.90 | 7.11 | 0.13 | 0.50 | 7.19 | 43.00 |
|  | Apidae | 6.29 | 0.61 | 0.00 | 0.25 | 0.06 | 0.50 |
|  | Tenthredinidae | 4.38 | 0.00 | 0.00 | 0.13 | 0.11 | 1.50 |
|  | Halictidae | 0.38 | 0.11 | 0.00 | 0.13 | 1.69 | 4.00 |
|  | Formicidae | 0.19 | 0.00 | 0.06 | 0.00 | 0.06 | 0.00 |
|  | Megachilidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 |
|  | Pompilidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 |
| Diptera | Syrphidae | 3.90 | 1.22 | 0.38 | 4.50 | 3.71 | 24.50 |
|  | Empididae | 2.95 | 0.17 | 0.00 | 0.00 | 6.63 | 7.50 |
|  | Scathophagidae | 0.05 | 0.00 | 0.00 | 0.13 | 0.11 | 14.00 |
|  | Calliphoridae | 0.14 | 0.00 | 0.00 | 0.13 | 0.73 | 6.50 |
|  | Anthomyiidae | 0.29 | 0.00 | 0.00 | 0.63 | 0.22 | 5.00 |
|  | Sciariidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.39 | 3.50 |
|  | Bibionidae | 0.19 | 0.28 | 0.00 | 0.00 | 0.06 | 2.00 |
|  | Ephydridae | 0.48 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 |
|  | Conopidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.28 | 0.50 |
|  | Muscidae | 0.00 | 0.00 | 0.00 | 0.13 | 0.06 | 2.00 |
|  | Sarcophagidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.50 |
|  | Tipulidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
|  | Bombyliidae | 0.10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
|  | Chironomidae | 0.00 | 0.00 | 0.00 | 0.25 | 0.00 | 0.00 |
|  | Agromyzidae | 0.00 | 0.00 | 0.00 | 0.13 | 0.00 | 0.00 |
|  | Chloropidae | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 |
|  | Phoridae | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 |
|  | Tachinidae | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

3.2 | Collection of insects using sticky traps

Insects captured using sticky traps are presented in Table 2. The families Andrenidae (Hymenoptera), Anthomyiidae (Diptera) and Cecidomyiidae (Diptera) shared large proportions of trap catches in 2019 and 2020. Relative frequencies of the collected insects fluctuated not only between years, but also among weeks in a year. It is noteworthy that this method might be unsuitable for trapping of some insect families, such as Apidae and Syrphidae, because of little trap catches of them.
3.3 | Contributions of flower-visiting insects to pollination

Access restriction effects of flower-visiting insects on fruit-set ratios are presented in Figures 1 and 2. The fruit-set ratio in the 3.5M treatment was significantly higher than that of either the 0M or 2M treatment in Tottori and central Kumamoto (p < .05). Significant differences in fruit-set ratios were observed between the 3.5M and 0M treatments in both 2019 and 2020 in Tochigi. The fruit-set ratio in the OP treatment was higher than that in the 3.5M treatment in all surveys, although the difference was not significant (p > .05). The increase in the fruit-set ratio in HPO treatment relative to OP treatment was not consistent between two surveys in Tottori and central Kumamoto. The fruit-set ratio in the 2M treatment was higher than that in the 0M treatment in Tottori in 2020 (p < .05).

Access restriction effects of flower-visiting insects on seed numbers are presented in Figure 2. Seeds were fewer in the 0M and 2M treatments than in either the 3.5M, OP or HPO treatment in Tottori (p < .05). In central Kumamoto, seed numbers in the 2M treatment were smaller than those in the 3.5M, OP and HPO treatments, although the difference was not significant (p > .05). No significant differences in seed number were found among the 2M, 3.5M and OP treatments in Tochigi. No seed was obtained in the 0M treatment in central Kumamoto and Tochigi (data not shown).

The number of pollen grains on the body surfaces of flower-visiting insects is presented in Table 3. Insects assigned to the families Andrenidae, Apidae, Halictidae, Syrphidae, Bibionidae and Muscidae carried large numbers of Pyrus pollen grains on their body surfaces. Some insect species, such as Andrena semirugosa brassicae Hirashima and Lasioglossum proximatum Smith, were commonly observed in the three regions. No such common pollinator species was observed for dipterans.

Head and thorax widths of the flower-visiting insects are presented in Table S1. Of the eight Andrenid species, five showed both widths smaller than 3.5 mm, as did 4 of 11 syrphid species. All species assigned to the families Halictidae, Bibionidae and Muscidae had both widths smaller than 3.5 mm. The families, Andrenidae, Halictidae, Anthomyiidae, Empididae, Calliphoridae, Bibionidae, Fanniidae, Sciaridae, Phoridae and Stratiomyidae included species with both widths smaller than 2 mm.

### Table 2

| Order | Suborder or Superfamily | Family | Species | No. of insects captured |
|-------|-------------------------|--------|---------|-------------------------|
|       |                         |        |         | 2019 Apr 12-19 | 2019 Apr 19-26 | 2020 Mar 26-Apr 2 | 2020 Apr 2-9 |
| Hymenoptera | Andrenidae | Panurginus crawfordi | 40 | 24 | 6 | 21 |
|           | Andrenidae | Andrena spp. | 28 | 11 | 0 | 1 |
|           | Apidae |        | 0 | 0 | 0 | 1 |
| Diptera | Anthomyiidae |        | 69 | 72 | 15 | 48 |
|          | Cecidomyiidae |        | 15 | 16 | 51 | 17 |
|          | Chironomidae |        | 0 | 0 | 89 | 10 |
|          | Drosophilidae |        | 3 | 16 | 2 | 0 |
|          | Diastatidae |        | 0 | 0 | 6 | 2 |
|          | Muscidae |        | 2 | 2 | 0 | 1 |
|          | Mycetophilidae |        | 1 | 4 | 0 | 0 |
|          | Sciidae |        | 0 | 0 | 2 | 1 |
|          | Syrphidae |        | 2 | 0 | 0 | 1 |
|          | Chaoboridae |        | 0 | 2 | 0 | 0 |
|          | Milichiidae |        | 0 | 2 | 0 | 0 |
|          | Phoridae |        | 0 | 0 | 2 | 0 |
|          | Calliphoridae |        | 0 | 0 | 0 | 1 |
|          | Psychodiidae |        | 0 | 0 | 1 | 0 |
|          | Sphaeroceridae |        | 0 | 0 | 0 | 1 |
|          | Tipulidae |        | 0 | 0 | 1 | 0 |
|          | Chalcidoidea |        | 0 | 0 | 0 | 4 |
|          | Symphyta |        | 0 | 0 | 0 | 1 |
| Total |                   |        | 160 | 149 | 175 | 109 |
3.4 | Fruit-set ratio in the OP treatment at open-pollinated and hand-pollinated orchards

Fruit-set ratio in the OP treatment is shown in Table 4, including the data presented in Figure 1. Results showed that fluctuation of year-to-year fruit-set ratios was less pronounced in open-pollinated orchards (Tochigi and southern Kumamoto) than in orchards in Tottori and central Kumamoto.

4 | DISCUSSION

Our results show that some families, such as Andrenidae, Apidae, Halictidae and Syrphidae, were commonly found with noticeable quantities in the three regions (Table 1). In addition to hymenopterans, importance of dipterans in pollination services has been recognized (Garibaldi et al., 2013; Orford et al., 2015; Rader et al., 2016; Symank et al., 2008). In fact, some dipteran groups are used as pollinators in greenhouses: for example, *Eristalis* sp. (Syrphidae) for peppers (Jarlan et al., 1997) and *Musca* sp. (Muscidae) and *Calliphora* sp. (Calliphoridae) for umbelliferous plants (Clement et al., 2007). Reportedly, insects assigned to the families Andrenidae, Apidae and Halictidae, and Syrphidae were abundant at Japanese pear orchards in Ishikawa (N36°E136°) and Fukushima (N37°E140°), Japan (Kumakura et al., 1973; Okabe, 1973). To examine the presence of key species on Japanese pear pollination, insects collected in 2020 were subjected to species identification based on their morphological characteristics. Results demonstrated that, excluding *A. mellifera*, two hymenopteran species, *An. semirugosabrassicae* (Andrenidae) and *L. proximatum* (Halictidae), were commonly observed in the three regions (Table 3). However, they did not constitute large proportions in any region. These results suggest that no key species exists for pollination services in Japanese pear orchards.

Our access restriction experiment in Japanese pear orchards showed a primary contribution to pollination services of insects that had passed through bags with 3.5 mm mesh. The families Andrenidae, Halictidae, Syrphidae, Anthomyiidae and Empididae included species, possibly accessible to the flowers in the 3.5M treatment, with head and thorax widths smaller than 3.5 mm (Table S1). In addition, *A. mellifera* (Apidae) and some species assigned to the families Andrenidae and Syrphidae with both widths larger than 3.5 mm (Table S1) might also contribute to pollination services because fruit-set ratios in the OP treatment were higher than that in the 3.5M treatment (Figure 1), but not significantly (*p* > .05). The six families included species harbouring large quantities of *Pyrus* pollen grains on their body surfaces (Table 3). Consequently, we concluded that the six families are the main components (hereinafter designated as small insects) responsible for pollination services.

Seed set has been recognized as an important indicator of fruit development: it influences fruit size, shape and eventually marketability (Brookfield et al., 1996; Buccheri & Di Vaio, 2005; Fountain et al., 2019; Garratt et al., 2014, 2016; Matsumoto et al., 2012; Monzón et al., 2004; Volz et al., 1996). Increased fruit-set ratios and seed set in the 2M treatment relative to the 0M treatment were observed in Tochigi (Figures 1 and 2). This finding suggests the presence of minute insects with head and thorax widths smaller than 2 mm, with involvement in pollination services at the study site. Some species assigned to the families Halictidae, Anthomyiidae and Empididae might have contributed to the increase. Reportedly, some gall midge species (Cecidomyiidae) are involved in pollination of *Artocarpus* plants (Moraceae) (Gardner et al., 2018; Sakai et al., 2000). In Tochigi, insects assigned to Cecidomyiidae were collected using sticky traps in large numbers in 2019 and 2020 (Table 2). In addition, *Zygophora* flies (Drosophilidae) are known to be involved in pollination of *Dracula* orchids in Andean cloud forests (Endara et al., 2010). The functional significance of these very small insects in pollination demands future investigation.

One index representing the pollination potential of flower-visiting insects is the number of pollen grains on the insect body surfaces (Devoto et al., 2011; Forup et al., 2008; Lopezaraiza-Mikel et al., 2007). Our surveys showed that insects assigned to the families Andrenidae, Apidae, Halictidae and Syrphidae harboured large quantities of *Pyrus* pollen (Table 3). Reportedly, stigma contact and visitation rates (flowers/ min) of pollinators in combination with the number of pollen grains affected fruit-set and seed-set of pear (Alarcón, 2010; Monzón et al., 2004; Popic et al., 2013). To evaluate the pollination potential of the small insects observed at the study sites in the three regions, their stigma contact rate and pollinating effectiveness must be examined in future studies. Additionally, the pollen grains deposited on a stigma must be quantified, as suggested by King et al. (2013).

The relative importance of specific pollinator species might change from year to year or season to season because populations of insect species show temporal fluctuation (Symank et al., 2008). As one might expect, the relative contributions of specific pollinator species for pollination services are presumed to change over time. Collection of flower-visiting insects of Japanese pear showed non-negligible differences in species composition and quantity between 2018 and 2019 (Table 1). In addition to the year-to-year fluctuation, data of sticky traps in Tochigi showed weekly fluctuations of species composition of various insect groups, including the families Andrenidae and Anthomyiidae (Table 2). These results suggest that pollination services conferred by specific pollinator species are fluctuating continuously in Japanese pear orchards. However, the fruit-set ratio in the OP treatment fluctuated less in the open-pollinated orchards in Tochigi and southern Kumamoto than in the hand-pollinated orchards in Tottori and central Kumamoto in our surveys conducted in 2019–2021 (Table 3). These results suggest that mutual complementarity among diverse pollinator species might be a fundamentally important mechanism for open insect pollination in Japanese pear orchards.

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# Table 3
Number of pollen grains on body surfaces of insects collected in Japanese pear orchards

| Order        | Family      | Species                              | Tochigi | No. of Rosaceae pollen (Mean ± SD) | No. of pollen other than Rosaceae (Mean ± SD) |
|--------------|-------------|--------------------------------------|---------|------------------------------------|---------------------------------------------|
| Hymenoptera  | Andrenidae  | Andrena benefica                     | 2       | 1,203 ± 420                        | 1,017 ± 1,116                               |
|              |             | Andrena dentata                      |         |                                    |                                             |
|              |             | Andrena japonica                     |         |                                    |                                             |
|              |             | Andrena kaguya                       | 9       | 5,557 ± 7,331                      | 255 ± 261                                   |
|              |             | Andrena luridiloma                   | 1       | 438                                | 0                                           |
|              |             | Andrena sasakii                      |         |                                    |                                             |
|              |             | Andrena semirugosa brassicae         | 3       | 13,157 ± 15,706                    | 529 ± 691                                   |
|              | Apidae      | Apis cerana japonica                 | 2       | 883 ± 663                          | 268 ± 175                                   |
|              |             | Apis mellifera                       | 13      |                                    |                                             |
|              | Halictidae  | Lasioglossum pallilomum              | 2       | 1,200 ± 552                        | 55 ± 63                                     |
|              |             | Lasioglossum proximatum              | 4       | 13,757 ± 13,553                    | 887 ± 1,134                                 |
|              |             | Lasioglossum sibiriacum              | 1       | 516                                | 30                                         |
|              |             | Lasioglossum vulsum                  | 2       |                                    |                                             |
| Tenthredinidae|             | Pachyproatis sp.                     | 6       | 1,688 ± 1,568                      | 112 ± 101                                   |
| Diptera      | Anthomyiidae| Delia platura                        | 7       | 4,574 ± 4,437                      | 24 ± 3                                      |
|              | Bibionidae  | Bibio japonica                       |         |                                    |                                             |
|              |             | Bibio holomarus                      |         |                                    |                                             |
|              | Calliphoridae| Stomorhina discolor                 |         |                                    |                                             |
|              |             | Calliphora nigribarbis               | 1       | 4,458                              | 30                                         |
|              |             | Hemipyrellia ligurriens              | 1       | 3,138                              | 14                                         |
|              |             | Calliphora vicina                    |         |                                    |                                             |
|              |             | Melinda sinensis                     | 1       | 0                                  | 6                                          |
|              | Empididae   | Empis (Polyblepharis) compsoygne     | 5       | 510 ± 503                          | 4 ± 3                                       |
|              |             | Empis (Anacrostichus) sp.1           | 2       | 351                                | 0                                          |
|              |             | Empis (Anacrostichus) sp.2           | 8       | 131 ± 146                          | 2 ± 4                                       |
|              | Fannidae    | Fannia prisca                       | 2       | 114 ± 59                           | 6 ± 8                                       |
|              |             | Fannia sp.                           | 5       | 4,241 ± 3,608                      | 55 ± 59                                     |
|              | Muscidae    |                                     | 1       | 48                                 | 30                                         |
|              | Phoridae    |                                     | 156     |                                    |                                             |
|              | Scleridae   |                                     | 1       | 156                                | 24                                         |
|              | Stratiomyiidae| Allognosta flavofemoralis          | 1       | 132                                | 6                                          |
|              |             | Actina jezoensis                     |         |                                    |                                             |
|              | Syrphidae   | Eristalis cerealis                   | 12      | 6,654 ± 8,093                      | 335 ± 318                                   |
|              |             | Syrphus torvus                       | 1       | 3,588                              | 174                                        |
|              |             | Eristalis tenax                      | 1       | 10,248                             | 390                                        |
|              |             | Episyrphus balteatus                 |         |                                    |                                             |
|              |             | Helophilus eristaloideus             | 6       | 5,198 ± 9,853                      | 254 ± 274                                   |
|              |             | Melanostoma mellinum                | 1       | 2,310                              | 78                                         |
|              |             | Ferdinandea cuprea                  | 1       | 1,458                              | 36                                         |
|              |             | Betasyrphus serarius                 |         |                                    |                                             |
|              |             | Cheilosia sp.                        |         |                                    |                                             |
|              |             | Eupeodes corollae                    |         |                                    |                                             |
|              |             | Eristalinus quinquelineatus          |         |                                    |                                             |

Abbreviations: n, No. of insects examined.
| Tottori      | Central Kumamoto                      |
|--------------|--------------------------------------|
| **n**        | **No. of Rosaceae pollen (Mean ± SD)** | **No. of pollen other than Rosaceae (Mean ± SD)** |
|              | **No. of pollen other than Rosaceae (Mean ± SD)** |                                |
| 4            | 19,050 ± 12,551                      | 349 ± 281                     |
| 1            | 1,578                                | 36                            |
| 3            | 9,507 ± 7,370                        | 603 ± 663                     |
| 1            | 2,136                                | 168                           |
| 2            | 9,350 ± 10,310                       | 515 ± 516                     |
| 14           | 127 ± 161                            | 4 ± 6                         |
| 3            | 6,402 ± 9,097                        | 534 ± 686                     |
| 1            | 2,922                                | 36                            |
| 1            | 12                                   | 0                             |
| 1            | 1,362                                | 18                            |
| 1            | 3,504                                | 480                           |
| 2            | 1,446 ± 1,375                        | 33 ± 13                       |
| 1            | 372                                   | 78                            |
| 1            | 2,184                                | 24                            |
|              | 1                                    | 3,834                         | 0                              |
|              | 1                                    | 6,108                         | 132                           |
|              | 2                                    | 609 ± 776                     |                                |
|              | 1                                    | 2,988                         | 0                              |
|              | 4                                    | 11,962 ± 7,785                | 142 ± 68                      |
|              | 2                                    | 1,200 ± 552                   | 55 ± 63                       |
|              | 4                                    | 2,033 ± 791                   | 21 ± 16                       |
|              | 2                                    | 5,525 ± 7,415                 | 42 ± 42                       |
|              | 14                                   | 63 ± 67                       | 8 ± 11                        |
|              | 3                                    | 210                           | 7                             |
|              | 5                                    | 1,206 ± 1,594                 | 10 ± 16                       |
|              | 2                                    | 282 ± 229                     | 6 ± 8                         |
|              | 1                                    | 4,344                         | 0                             |
|              | 15                                   | 157 ± 158                     | 11 ± 27                       |
|              | 1                                    | 408                           | 0                             |
|              | 1                                    | 507                           | 3                             |
|              | 1                                    | 186                           | 0                             |
|              | 1                                    | 900                           | 6                             |
|              | 1                                    | 138                           | 132                           |
|              | 1                                    | 150                           | 72                            |
|              | 1                                    | 1,308                         | 36                            |
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CONFLICT OF INTERESTS
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
SS conceived research and wrote the manuscript. KK, YF, KN, MK, ST and NS conducted experiments. SN, MS and TM identified insects. MT conducted statistical analyses of the data. All authors read and approved the manuscript.

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The data that support the findings of this study are available in (Sonoda, 2021a, 2021b) figshare at https://doi.org/10.6084/m9.figshare.16606715.v1 and https://doi.org/10.6084/m9.figshare.1660694.v1

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